The Role of Dectin-2 during infection with Mycobacteria that are able to survive in Macrophages

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Abstract

Tuberculosis caused by *Mycobacterium tuberculosis*, is one of the leading causes of death on a global scale. Multi-drug resistant and extremely drug resistant *M. tuberculosis* is a world-wide health threat. Pathogenic mycobacteria can survive because they are able to manipulate the host immune response in several ways. In a previous RNA sequencing study, several genes, including *Clec4n* was significantly upregulated in bone marrow-derived macrophages (BMDMs) infected with slow-growing mycobacteria (H37Rv and *M. bovis* BGC), compared to fast-growing mycobacteria (*M. smegmatis*) that were not able to survive. The experimental approach has been done as described in (Leisching et al., 2016a) and (Leisching et al., 2016b).

In this study, the mouse gene *Clec4n* was studied to determine its role in mycobacterial infection. The mouse *Clec4n* gene is translated into the Dectin-2 receptor that is able to recognize mycobacteria and initiate an immune response by binding to Mannose-capped lipoarabinomannan (Man-LAM). BMDMs were infected with pathogenic (H37Rv) and non-pathogenic (*M. smegmatis* and *M. bovis* BCG) mycobacteria. At a 12h and 96h time point, RNA and protein was extracted for qPCR (molecular technique that monitors the amplification of a targeted DNA molecule) and western blots. To achieve a better understanding of the role that Dectin-2 plays during infection of pathogenic mycobacteria in mouse macrophages, the Dectin-2 receptor was blocked with an anti-Dectin-2 antibody, to observe the effects on survival by Colony forming unit counts. In addition, the amount of release of key cytokines (TNFα and IL-10) by mouse macrophages was determined by Enzyme-linked immunosorbent assay (ELISA) (solid-phase enzyme immunoassay that detect the presence of a ligand in a liquid sample using antibodies directed against the protein to be measured).

According to the qPCR results, the induced expression of *Clec4n* was only an early response, as decreased expression was determined at 96h post infection by *M. bovis* BCG and H37Rv. When the CFUs of 12h of infection was compared to the CFUs of 96h there was not a change in the survival or growth of the mycobacteria. Its results can indicate that Dectin-2 may influence the percentage uptake of H37Rv but not

the survival. The ELISA results showed that there was a significant increase in the production of TNFα , in the BMDMs infected with H37Rv and treated with the anti-dectin-2 antibody compared to the BMDMs infected with H37Rv but no antibody treatment, that suggests that binding of the antibody stimulated a higher production of TNFα. When the Dectin-2 antibody binds to the Dectin-2 receptor, mycobacteria binds to other receptors which initiate in a high production of TNF-α, that causes a high inflammatory response that leads to a lower survival of mycobacteria. To observe the response this effect has on the whole immune response, whole animal studies can be done. Gene silencing can be done with Dectin-2 to investigate the role it has on survival of pathogenic mycobacteria. Dectin-2 investigation can be done in human macrophages.

Uittreksel

Tuberkulose, veroorsaak deur *Mikobakterium Tuberkulose*, is een van die grootste oorsake van dood wêreldwyd. Patogeniese mikobakterieë het die vermoë om te oorleef in die gasheer, omdat dit die immuunsisteem van die gasheer op verskeie maniere kan manipuleer. In 'n vorige studie was 'n RNA volgorde studie gedoen op verskeie gene, insluitende die muis *Clec4n* geen, wat beduidend opgereguleer was in in die beenmurg afgeleide Makrofae (BMAM) geÏnfekteer met mikobakterieë (*M. tuberkulose* en *M. bovis* BCG), wat die vermoë het om te oorleef in die makrofae in vergelyking met die BMAMs wat geÏnfekteer is met mikobakterieë (*M. smegmatis*) wat nie kan oorleef in die makrofae nie. Die eksperimentele benadering was gedoen soos beskryf in (Leisching et al., 2016a) en (Leisching et al., 2016b).

In die studie word die muis *Clec4n* geen bestudeer om die vedere rol te bepaal wat dit speel tydens patogeniese mikobakterium infeksies. Die muis *Clec4n* geen word getransleer in die Dectin-2 reseptor, wat mikobakterieë herken tydens infeksie, deur aan die Man-Lam van die mikobakterieë te bind en 'n imuunrespons te inisieër. MBAMs was geÏnfekteer met H37Rv, *M. bovis* BCG en *M. smegmatis*. RNS en protëine was ge-isoleer na 'n 12 - en 96 uur infeksie tyd punt vir kwalitatiewe polimerase kettingreaksie (PKR) ('n molekulêre tegniek wat die amplifikasie van 'n teiken DNS molekule monitor) en westerse blots. Om die rol wat Dectin-2 speel tydens infeksie van patogeniese mikobakterieë beter te verstaan, was die Dectin-2 reseptor geblok met n anti-dectin-2 teenliggaam, om die effek te observeer. Daar was geobserveer of die intersellulêre oorlewing van patogeniese mikobakterieë (H37Rv) verander wanneer die Dectin-2 reseptor geblok word (deur te observeer of die kolonievormende eenhede verlaag), asook of die konsentrasie (pg per ml) van sitokienes (TNFα en IL-10) met behulp van ELISA (soliede-fase ensiem immunotoets wat die aanwesigheid van 'n vloeistof monster opspoor deur teenliggame wat aan die protein kan bind, meet) verander (veral verlaag word), wanneer die dectin-2 reseptor geblok is.

Volgens die resultate van die kwalitatiewe PKR, speel *Clec4n* ‘n moontlike rol in die oorlewing van *M. bovis* BCG en H37Rv na ‘n 12 uur infeksie, weens die feit dat daar ‘n beduidende opregulasie van *Clec4n* in die BMAMs geÏnfekteer met *M. bovis* BCG en H37Rv in vergelyking met BMAMs geÏnfekteer met *M. smegmatis* was. Wanneer die KVE na 12 uur van infeksie vergelyk was met die KVE na 96 uur van infeksie, was daar geen verandering in die oorlewing of groei van die mikobaterieë. Die resultate dui dat Dectin-2 ‘n invloed het op die persentasie opname van H37Rv maar nie ‘n invloed op die oorlewing nie. In die ELISA resultate was daar gevind dat daar ‘n beduidende hoër produksie van TNFα sitokienes is in die BMAMs, geÏnfekteer met H37Rv en anti-dectin-2 teenliggaam behandeling was as in die BMAMs, geÏnfekteer met H37Rv en geen behandeling nie. Dit dui daarop dat binding met die teenliggaam ‘n hoër produksie van TNFα sitokines gelewer het. Die Dectin-2 teenliggaam bind aan die Dectin-2 reseptor, as gevolg hiervan gaan bind die mikobakterieë aan ander reseptors wat die produksie van TNF-α sitokienes verhoog, wat ‘n hoë inflammatoriese reaksie veroorsaak en kan lei tot die verlaging van die orrlewing van mikobakterieë. Om die effek van die Dectin-2 teenliggaam binding as ‘n geheel te observer in die immuunsisteem kan heel dier studies gedoen word. Geen uitdowing kan gedoen word op Dectin-2 om die rol daarvan in die oorlewing van patogeniese mikobakterieë te ondersoek. Die ondersoek van Dectin-2 kan gedoen word in mens makrofae.

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List of Abbreviations

%	Percentage
˚C	Degree Celsius
µl	Microlitre
pg	Picogram
BCG	Bacillius Calmette Guérin
BCL10	B-Cell Lymphoma 10
B-2-M	Beta-2-Microglobulin
BMDM	Bone Marrow Derived Macrophages
CARD	Capase Recruitement Domain
cDNA	Complementary DNA
CFU	Colony Forming Units
CR	Complement Receptor
CRD	Carbohydrate Recognition Domain
CTLR	C-Type Lectin Receptor
CFS	Colony Stimulating factor
DC-SIGN	DC-specific intercellular adhesion molecule-3 grabbing non-integrin
ELISA	Enzyme Linked Immunosorbent Assay

FBS	Fetal Bovine Serium
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gDNA	Genomic DNA
h	Hour
IL-10	Interleukin 10
JAK-STAT	Janus Kinase Signal Transducer and Activator of Transcription
kDa	Kilo Daltons
Man-LAM	Mannose-capped lipoarabinomannan
MALT	Mucasa Associated Lymphoid Tissue
MHC	Major Histocompatibility Complex
Min	Minutes
ml	Millilitre
MIQE	Minimum Information for Publication for Quantitative PCR Experiments
ng	Nanogram
nm	Nanometer
MOI	Multiplicity of Infection
MR	Mannose Receptor
OADC	Oleic Albumin Dextrose Catalase

OD	Optical Density
PAMP	Pathogen associated molecular patterns
PBS	Phosphate Buffer Saline
PDIM	Phthiocerol Dimycocerosate
P13P	Phosphatidylinositol 3 phosphate
PKC	Protein Kinase C
PMA	Phorbol 12 Myristate 13-Acetate
PRR	Pattern Recognition Receptors
qPCR	quantitative Polymerase Chain Reaction
RIN	RNA Integrity Number
RIPA	Radioimmunoprecipitation Assay
RPM	Rounds per Minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SSF	Syringe Settle Filtrate
Syk	Spleen Tyrosine Kinase
TB	Tuberculosis
TLR	Toll Like Receptor
TNFα	Tumor Necrosis Factor Alpha

UBC	Ubiquitin C

CHAPTER 1: Literature review

1.1 Background

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a pandemic throughout the world. Many TB patients have been cured, but there are many that have multidrug resistant and extremely drug resistant TB that conventional drugs can not cure. It is therefore crucial to look at alternative ways to combat the disease such as targeting the host immune response and developing host directed therapeutics. Pathogenic mycobacteria are able to avoid bactericidal host defences and prevent from being eliminated by an active immune response (Eht and Schnappinger, 2009). In a RNA sequencing study several genes were identified to be significantly upregulated in bone marrow-derived macrophages (BMDMs) infected with mycobacteria (*M. tuberculosis* H37Rv, *M. bovis* BCG Bacillius Calmette Guérin) that are able to survive in the macrophages compared to mycobacteria (*M. smegmatis*) that are killed. In this study one of the identified genes, the mouse gene *Clec4n* was studied to determine its role in mycobacterial infection. The experimental approach has been done as described in (Leisching et al., 2016a) and (Leisching et al., 2016b).

1.2 Infection by *M. tuberculosis*

M. tuberculosis is spread through the air from an infected person to a healthy person. Once the bacterium has been inhaled, it enters the lungs and is taken up by alveolar macrophages and dendritic cells (Cooper, 2009). Alveolar macrophages recognize specific pathogen-associated molecular patterns (PAMPs). *M. Tuberculosis* is taken up by alveolar macrophages though a process called phagocytosis. After the macrophages have been infected, they release pro-inflammatory cytokines that recruit other immune cells such as neutrophils, monocytes and dendritic cells to the lungs. Infected dendritic cells migrate to the lymph nodes where they initiate an adaptive immune response (Stamm et al., 2015).

In an adaptive immune response, granulomas are formed. Activated T lymphocytes move to the site of infection and start to form a granuloma. The cells form a necrotic central core that gives the mycobacteria nutritional supplements and limit the spread of mycobacteria growth (Co et al., 2004).

Infected macrophages secrete pro-inflammatory cytokines such as Tumor necrosis factor α (TNFα), Interferon γ (IFNγ), interleukin-12 and-23 and chemokines (Sasindran and Torrelles, 2011). The initial interaction between mycobacteria and its host, determines the pathway and the outcome of the infection. Specific host receptors are able to recognise mycobacteria and initiate an innate immune response however some receptors that recognise *M. tuberculosis* cause no pro-inflammatory response and lead to *M. tuberculosis* survival (Sasindran and Torrelles, 2011).

Mycobacteria release antigens that are specific to it. These include lipomannan, lipoarabinomannan, and Mannose-capped lipoarabinomannan (ManLAM), lipoproteins, phthiocerol dimycocerosate (PDIM), and mycolic acids. These antigens are recognized by pattern recognition receptors. The main cell types involved in recognizing and intiating an immune response to *M. tuberculosis*, are macrophages and dendritic cells. (Stamm et al., 2015).

1.2.1 Pattern Recognition Receptors

The main surface receptors that recognize *M. tuberculosis* include Toll-like receptors, C-type lectin receptors and scavenger receptors.

Toll-like receptors (TLR) that are found on the surface of macrophages are TLR 1, 2, 4 and 8. TLRs recognize a wide variety of structures from *M. tuberculosis* (Kawai and Akira, 2010). Different components of *M. tuberculosis* act with different TLRs. The TLRs that play a main role in recognizing *M. tuberculosi*s are TLR 2, 4, 8 and 9. TLR 2 can cause a strong pro-inflammatory response induced by the mycobacterium cell wall components such as lipoproteins (Harding and Boom, 2010).

Activation of TLRs leads to the activation of mitogen activated protein kinases (MAPKs), which initiate signalling cascades that cause the expression of the cytokine TNF α and several chemokines. It was found that this pathway could be exploited by pathogenic mycobacteria to promote survival of the mycobacteria within macrophages. ManLAM from *M. tuberculosis* inhibits the activation of MAPK in human monocytes (Knutson et al., 1998).

C-Type lectin receptors are plasma membrane molecules that are able to recognize carbohydrate moieties on the surface of pathogens. C-type lectin receptors that are able to recognize *M. tuberculosis* include mannose receptor (MR), DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN), Mincle, Dectin-1 and Dectin-2 (Killick et al., 2013).

The mycobacterial cell envelope consists of high levels of mannose biomolecules that act as ligands for the mannose receptors on macrophages that contribute to *M. tuberculosis* pathogenesis. It has been found that the mannose receptor negatively regulates the macrophage pro-inflammatory response (Torrelles and Schlesinger, 2010).

All mononuclear monocytes express complement receptors that regulate phagocytosis. *M. tuberculosis* have polysaccharides on their surface that bind to the Complement receptor 3 lectin domain, which moderates *M. tuberculosis* uptake by macrophages (Fenton et al., 2005).

Complement receptor 3 (CR3) plays an important role in recognizing mycobacteria. For phagocytosis of mycobacteria though CR3, it requires the plasma membrane steroid cholesterol at the site of entry. Cholesterol increases the viscosity of the membrane that is in contact with the hydrophobic mycobacterial cell wall and thus increases the uptake by phagocytosis. When CR3 is inhibited by antibody binding or the blocking of its lectin site, it leads to reduction of mycobacterial uptake by phagocytosis (Gatfield and Pieters, 2000).

CR4 is highly abundant in cells that take up *M. tuberculosis* and is also known to play an important role in the early stages of *M. tuberculosis* infection (Hirsch et al., 1994).

1.2.2 Cytokines and Chemokines

The immune response is regulated by polypeptides known as cytokines. The main cytokines involved are Tumor necrosis factor-α, Interleukin 12 (IL-12) and Interferons (Sasindran and Torrelles, 2011).

Tumor necrosis factor-α (TNF-α) is an autocrine cytokine secreted from macrophages, dendritic cells and T-cells. TNF plays a role in granuloma formation and regulates some adhesion molecules that are crucial for granuloma formation. When TNF α is uncontrolled, it leads to increased *M. tuberculosis* replication (Bean et al., 1999).

Interleukin 12 (IL-12) is induced by macrophages and dendritic cells when they are activated though microbial TLR ligands. It plays a role in both innate and adaptive immune responses against *M. tuberculosis*. IL-12 activates the Janus Kinase Signal Transducer and Activator of Transcription (JAK-STAT) pathway that leads to the production of INF-γ, which in turn causes the production CD4 cells (Méndez-Samperio, 2008).

The interferon family is divided into two types depending on its structure, function and cell origin. Type 1 include IFN-α and IFN-β and is produced through immune receptors through different cell types. Type 2 includes IFN-γ and is produced when T lymphocyte and natural killer cells are stimulated. When macrophages are infected with *M. tuberculosis*, IFN-α and IFN-β are produced, which stimulates the production of $CD8^+$ and $CD4^+$ T cells (Cho et al., 2002) and thus enhances the adaptive immune response.

The type 1 interferon response can contribute to host susceptibility because the inflammatory response is overwhelming to the cell. IFN-γ is considered an important cytokine in the control of *M. tuberculosis*. It enhances the macrophages to produce pro-inflammatory cytokines, up-regulates surface expression of cytokines, chemokines, MHC 1 and MHC 2 molecules to allow macrophages to presents antigen to T- cells (Manca et al., 2001)

1.3 *M. tuberculosis* evades anti-mycobacterium mechanisms in the macrophage

When a host is infected with a non-pathogenic mycobacterium such as *M. smegmatis*, the non-pathogenic mycobacterium is phagocytosed by macrophages (Figure 1.1). The phagosomes become acidified and fuse with lysosomes to become phagolysosomes. This environment is fatal to *M. smegmatis* and other non-pathogenic mycobacteria, however pathogenic mycobacteria such as *M. tuberculosis*, have counter mechanisms that enable them to prevent the fusion of phagosomes with lysosomes, thus enabling them to multiply and eventually be released to infect other macrophages and cause disease in the host (Bohsali et al., 2010).

ManLAM has been found to block phagosome maturation. ManLAM blocks the PI3P (phosphatidylinositol 3 phosphate)-dependant pathway that is involved in the transport of cargo between the trans-Golgi network and phagosomes, which is a transport step in phagosome maturation. A high Ca^{+2} concentration is needed in the cytosol of the macrophage for PI3P to bind to the phagosome prevented by ManLAM. ManLAM might also prevent PI3P from interacting with phagosomes by binding to proteins containing domains that PI3P would normally bind to (Vergne et al., 2004).

Pathogenic mycobacteria can survive because they are able to manipulate the host immune response in several ways (Figure 1.1). In a study (MCGARVEY et al., 2004) that monitored the gene expression of macrophage genes infected with pathogenic mycobacteria (*M. tuberculosis* and *M. avium*) and non-pathogenic mycobacteria (*M. smegmatis*), a difference in gene expression was observed between pathogenic and non-pathogenic mycobacteria. A Higher expression of genes that induce apoptosis was found in cells infected with *M. tuberculosis* and *M. avium*. Cathepsin D and AP2M1, which are genes involved in lysosome acidification were suppressed in *M. tuberculosis* and *M. avium* in contrast to *M. smegmatis* (MCGARVEY et al., 2004)

The generation of reactive oxygen and nitrogen species also play a role in microbe degradation. Nitric oxide synthesis (iNOS) is a cytosolic enzyme that catalyses the conversion of L-arginine to L-cittruline and nitric oxide. Mycobacteria are able to prevent binding of iNOS to the phagosomes causing a reduction of nitric oxide production (MacMicking et al., 1997). The envasion of host killing is influenced by the receptor/s employed by mycobacteria upon entry into macrophages.

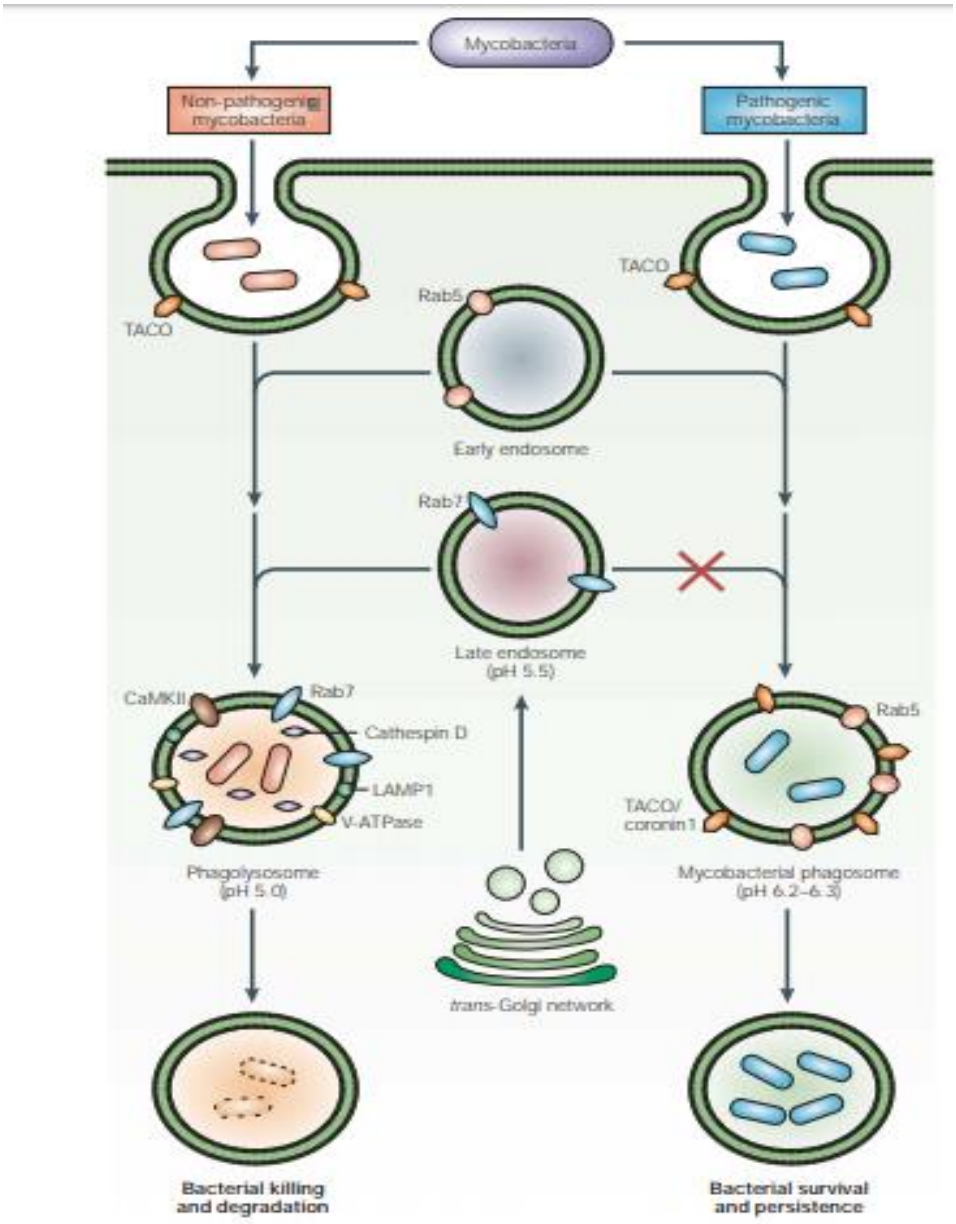

Figure 1.1: There are different pathways of pathogenic and non-pathogenic mycobacteria when it infects the host. Pathogenic mycobacteria such as *M. tuberculosis* have found mechanisms to escape the fusion of the phagosomes with the lysosomes and in that way manages to survive. Image adapted from (Koul et al., 2004).

1.4 Dendritic cell associated C-type lectin 2 Family

Dectin-2 is part of the C-type lectin-like receptor family that play important roles in immunity and homeostasis. The Dectin-2 family, also known as dendritic cell-associated C-type lectin 2, includes Dectin-2, blood dendritic cell antigen 2, dendritic cell immunoactivating receptor, dendritic cell immunoreceptor, C-type lectin superfamily 8 and macrophage inducible C-type lectin. These receptors consist of an extracellular conserved C-type lectin domain and they can mediate intracellular signalling either directly through integral signalling domains or indirectly by associating with signalling adaptor molecules. These receptors are able to recognize a variety of endogenous and exogenous ligands and function as pattern recognition receptors for different classes of pathogens that include fungi, bacteria and parasites that will lead to both an innate and adaptive immune response (Kerscher et al., 2013).

The C-type lectin receptors can be found in either membrane bound or soluble forms. These receptors consist of at least one carbohydrate recognition domain, also known as a C-type lectin domain that is formed by disulphide bonds that are found between highly conserved cysteine residues. Many of the CTLRs are found to be multivalent. By being able to initiate intracellular signalling pathways, they are able to meditate cellular responses (Kerscher et al., 2013)

The genes that encode the dendritic cell-associated C-type lectin-2 family, are grouped on the telomeric region of the natural killer gene cluster in mice in chromosome 6 and in human in chromosome 12. These CTLRs are type 2 transmembrane receptors that consist of a single extracellular conserved C-type lectin domain (Graham and Brown, 2009).

1.4.1 Dectin-2

Dectin-2 is expressed on a variety of myeloid cells. This includes tissues of macrophages, neutrophils and DCs. The *Clec4n* mouse gene, is translated into the Dectin-2 receptor. Dectin-2 is also expressed at low levels on peripheral blood monocytes although the expression can be highly upregulated during inflammation.

Human Dectin-2 has been found on monocytes and DCs and have also been expressed by lymphocytes. The human Dectin-2 gene, *Clec6A* is found on chromosome 12 and the mouse Dectin-2 gene is found on chromosome 6. (Taylor et al., 2005)

Dectin-2 consists of an extracellular C-terminal C-type carbohydrate-recognition domain (CRD) that is linked to a transmembrane domain and a N-terminal cytoplastic domain (Figure 2.1). This receptor does not have any signalling motifs in its cytoplastic membrane. Dectin-2 has a short cytoplasmic tail that binds with Fc receptor γ subunit (FcRγ), which consists of an immunotyrosine activation motif that interacts with Syk kinase, which leads to a secretion of several cytokines leading to a Th17 response. The binding of the Dectin-2 receptor to FcRγ is mediated by a membrane-proximal part of its short cytoplasmic tail (Sato et al., 2006a).

Dectin-2 plays a role as a pathogen recognition receptor that can recognize several pathogens. The most general ligands for mouse Dectin-2 are α-mannas, *M. tuberculosis*, house dust mite, Fungi (*Candidas*), $CD4^+$, $CD25^+$ and T-cell ligand. Signalling from Dectin-2 is mediated by Syk, PKCδ and CARD9-Bcl10-Malt1 (caspase recruitment domain-9-B-cell lymphoma translocation gene 1) pathway that leads to the production of a variety of cytokines and chemokines such as TNF-α, IL-2, IL-10, IL-23, IL-6 and IL-12. These cytokines leads to a Th1 and Th17 response (Sato et al., 2006a).

Dectin-2 has a classical CTLD that contains a mannose binding motif that binds structures with a high mannose content. Dectin-2 is capable of recognizing a number of pathogens including *M. tuberculosis* (Sato et al., 2006a).

1.5 Dectin-2 and its association with pathogens other than *M.tuberculosis*

Previous studies have found that Dectin-2 is able to recognize various pathogens. These include *Candida albicans*, *Saccharomyces cerevisiae*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, *Aspergillus fumigatus*, non-encapsulated *Cryptococcus neoformans*, *Microsporum audouinii*, *Trichophyton rubrum, Schistosoma mansoni* and house dust mite (Sato et al., 2006). Triggering of Dectin-2 induced a respiratory

burst (Gorjestani et al., 2011), and nucleotide-binding oligomerization domain-like receptor containing pyrin domains 3 (Nlrp3) inflammasome activation. The following includes more detail of how Dectin-2 plays a role in some of the above mentioned pathogens (Ritter et al., 2010).

Dectin-2 is able to recognize *C. albicans* through β-glucan. During infection with *C. albicans*, Dectin-2 regulates host inflammatory cytokine response and the development of adaptive immunity. Dectin-2 was found to preferably bind to hyphal rather than conidial components of *C. albicans*. Mice without this receptor where found to have a higher susceptibility to this infection (Sato et al., 2006).

The morbidity of *S. mansoni*, which is a human parasite, occurs through the $CD4^+$ T-cell mediated response to eggs that become trapped in the liver and intestinal tissue. Dectin-2 recognizes the parasitic worm, *S. mansoni* by its soluble schistosomal egg antigen (SEA). Dectin-2 stimulates an immune response through the Syk pathway. The Syk activity leads to Nlrp3 inflammasome which alters adaptive immune responses (Ritter et al., 2010).

House dust potently generates allergic inflammation. Extracts from the house dust mites, *Dermatophagoids farina* and *Dermatophagoids pteronys* and from the mold, *Aspergillus fumigates* stimulate the production of cysteinyl leukotrienes (cys-LTs) which are pro-inflammatory lipid mediators that cause bronchial smooth muscle constriction, vascular permeability and pulmonary inflammation in bronchial asthma. Transfection of each receptor in bone marrow mast cells revealed that only Dectin-2 mediates cys-LT production of these above named house dust mites and mold. Through the Dectin-2 mechanism, these allergens activate the immune cells to promote allergic inflammation (Barrett et al., 2009).

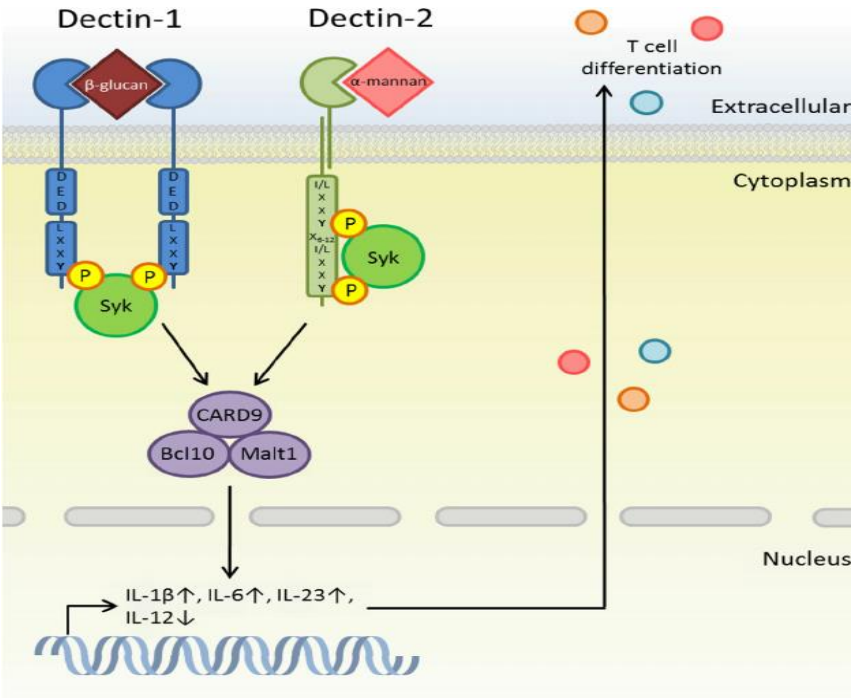

Figure 1.2: Dectin-2 contains a mannose binding motif that binds to mannose ligands. The FcRγ receptor is meditated by a membrane-proximal part of the Dectin-2 short cytoplasmic tail. Signalling from Dectin-2 is mediated by Syk, PKCδ and CARD9-Bcl10-Malt1 pathways. Cytokines that are expressed through this pathway leads to T cell differentiation. Image adapted from (Plato

1.6 Dectin-2 and its association with *M. tuberculosis*

M. tuberculosis contain a variety of immunomodulatory molecules on their cell walls. ManLAM is a major lipoglycan for *M. tuberculosis*. C-type lectin receptor Dectin-2 binds directly to Man-LAM. LAM consists of four components, which include mannosyl-phosphatidyl-myo-inositol anchor, a mannose backbone, arabinan domain and capping moieties (Misha et al., 2011). When Man-LAM binds to Dectin 2, a pro- and anti-inflammatory immune response is triggered in dendritic cells. Man-LAM can also cause a high T-cell mediated acquired immunity that leads to detrimental inflammation (Robinson et al., 2009).

When Man-LAM is recognized, it causes an inflammatory response that includes cytokine production in dendritic cells. It induces the expression of inflammatory cytokines that include MIP-2, TNF-α and IL-6 (Ariizumi et al., 2000).

Man-LAM also plays an immunosuppressive role. It induces the production of anti-inflammatory cytokine IL-10 in bone marrow-derived dendritic cells (BMDCs). There was no release of IL-10 cytokines that was induced by Man-LAM in BMDCs form Clec4n$^{-/-}$ BMDC mutant mice. With infection with *M. bovis* BCG the production of IL-2 and IL-10 was very low in BMDCs from Clec4n$^{-/-}$ mutants. *M. abscessus*, which has no mannose cap, was also not able to cause an expression in these anti-inflammatory cytokines. The results suggested that Dectin-2 plays an important role in the production of IL-2 and IL-10 in response to mycobacterium (Yonekawa et al., 2014).

Binding of Dectin-2 receptor both initiates pro- and anti-inflammatory responses. It is still unknown whether Dectin-2 receptor plays a protective or non-protective role towards Mycobacteria.

1.7 Problem Statement and Motivation

In a prior high-throughput RNA sequencing study, it was observed that the expression of several genes that included *Clec4n* (Dectin-2 gene) was significantly upregulated in mouse bone marrow-derived macrophages infected with slow-growing mycobacteria (H37Rv and *M. bovis* BGC) as compared to fast growing mycobacteria (*M. smegmatis*) that are not able to survive. Since conflicting reports about the pro- and anti-inflammatory responses induced by Dectin-2 exist, it remains unclear whether the engagement of Dectin-2 by mycobacteria influences the survival of pathogenic mycobacteria.

1.8 Hypothesis

The induced gene expression of *Clec4n* yields an increase in Dectin-2 on the cell surface and influences the survival of slow-growing mycobacteria (H37Rv) and *M. bovis* BCG in mouse bone marrow-derived macrophages.

1.9 Aim

To determine whether Dectin-2 influences the survival of slow-growing mycobacteria (H37Rv) in BMDMs.

1.10 Objectives

- To establish whether the gene expression of Dectin-2 in comparison with the different strains correspond with the results of the RNA-sequencing data.
- To assess the expression of the *Clec4n, TNF* α and *IL 10* gene at early (12h) and later (96h) time points in mouse bone-marrow-derived macrophages infected with slow-growing mycobacteria (H37Rv and *M. bovis* BCG) and fast-growing mycobacteria (*M. smegmatis*).
- To determine if the *Clec4n* gene is translated into the Dectin-2 protein after expression when infected with mycobacteria, at a 12h and 96h time point.
- To observe the difference in survival of the pathogenic mycobacteria (H37Rv) when blocking the Dectin-2 receptor with a specific antibody.
- To determine if blocking the Dectin-2 receptor affects the release of key cytokines (TNFα and IL-10) known to be induced by binding to Dectin-2.

CHAPTER 2: Materials and Methods

In order to observe the gene expression of Dectin-2 during infection of mycobacteria (H37Rv and *M.bovis* BCG) that are able to survive in BMDMs and mycobacteria(*M.smegmatis*) that are not able to survive, the following needed to be done first.

Mycobacteria (H37Rv, *M.bovis* BCG and *M.smegmatis*) were cultured in order for there to be stocks to infect the BMDMs with. Mycobacteria was titrated, so that the correct MOI (Multiplicity of Infection) could be calculated. BMDMs were cultured, differentiated and counted in order for the right MOI can be calculated. After infection RNA was extracted and converted to cDNA to be ready for qPCR. Protein was also extracted, and protein quantification was done to load the correct amount of protein for SDS-PAGE and western blot.

The number of cells were estimated for 96 well plates to calculate the right MOI. CFU counts were done and supernatant was collected for ELISAs.

2.1 Mycobacteria stocks

2.1.1 Culturing Mycobacteria

The three mycobacterium strains being non-pathogenic *M. smegmatis* and attenuated *M. bovis* BCG and the pathogenic H37Rv strain, were obtained from the Division of Molecular Biology and Human Genetics, Stellenbosch University. The mycobacteria were grown in Middlebrook 7H9 (Difco, Becton Dickson, USA) medium supplemented with 10% oleic acid albumin-dextrose catalase (OADC, Becton Dickson, USA) enrichment medium and 0.5 % glycerol (Merck Millipore, Germany) excluding Tween 80.

Slow growing mycobacteria which include H37Rv and BCG, were grown in the following way: A frozen stock vial of bacteria was thawed and syringed 10 times through a 25G needle (Becton Dickson, USA). The bacteria were inoculated into non-tween 80, Middlebrook 7H9 medium in two T25 flasks. The bacteria (0.5 ml) and

9.5 ml of Middlebrook 7H9 medium in each T25 flasks were placed in an incubator at 37˚C. The culture was grown to an OD_{600} (Optical Density) of 0.3 absorbance taken in an MRC spectro UV-16 spectrophotometer.

Each T25 flask was then split into five T25 flasks by adding 1 ml of starter culture and 9 ml of Middlebrook 7H9 medium into each new T25 flask. They were then placed in an incubator at 37˚C. The bacteria were grown to an OD_{600} of 0.4 absorbance. Each T25 flask was then split into two T25 flasks, which gave 20 T25 flasks in total. The flasks were placed in an incubator at 37˚C. The bacteria were grown to an OD_{600} of 0.4 absorbance.

The fast-growing mycobacteria included *M. smegmatis* and were grown in the following way: The OD_{600} was taken for the start culture. The initial culture started with 10 ml in a 100 ml Erlenmeyer flask. The culture started with an OD_{600} of 0.0025 absorbance. The bacteria were inoculated in Middlebrook 7H9 medium. The flask was incubated at 37˚C and shaker that showed visible swirling in a Lasec incubator. The OD_{600} was taken 12h after incubation. The bacteria were then taken up to 50 ml each in four 500 ml Erlenmeyer flasks with an OD_{600} of 0.0025 absorbance. After 12h of incubation, the OD_{600} did not exceed 0.4 absorbance.

2.1.2 Titration

The cultures were combined into four 50 ml tubes and were given 10 min for the clumps to settle down. The top 45 ml of each tube was transferred into a new tube. The tubes were centrifuged at 1500 rpm for 5 min in an Eppendorf 5810 R Centrifuge and the supernatant was discarded. Each pellet was resuspended in 5 ml of 7H9 medium and mixed well by pipetting. Several 1ml tubes were filled with 1 ml of culture and stored at - 80˚C.

Three vials of frozen bacteria stock (Three technical replicates) and a control added of Middlebrook 7H9 medium were used for titration. The bacteria were pipetted though a 1 ml tip ten times and then syringed ten times though a 25G needle. The clumps could settle out in the following times: 1 min for M. *smegmatis*, 30 Seconds for BCG and 10 min for H37Rv .The bacteria were added in a 15 ml tube which contained 4.250 ml of Middlebrook 7H9 medium and 750 µl of bacteria. The bacteria were filtered through a 5.0 µm pore

size filter (Merck, Darmstadt, Germany), each one separately. Dilutions of each three were made from $x10^{-1}$ to $x10^{-4}$ and 50 µl was plated out on a Middlebrook 7H11 (Difco, Becton Dickson, USA) agar split plate. The plates were incubated at 37˚C. After 3 days for *M. smegmatis* and 3 weeks for H37Rv and BCG the CFU was counted. An average was calculated between the 3 technical replicates which would be equal to the number of bacteria per ml.

2.2 Obtaining Murine Bone Marrow-Derived Macrophages

Bone marrow-derived macrophages were obtained from femur bone marrow. The femurs were from four to six-week-old female C57BL/6 mice and the mice were obtained from the Animal Facility, Stellenbosch University. Ethics for primary cultures obtain from mice were approved with protocol number, SU-ACUD14-00041. After the mouse femurs were obtained the bone marrow cells were extracted according to a protocol from Chantal De Chastellier (De Chastellier et al., 2009).

The cells obtained were grown in RPMI- 1640 (containing L-glutamine and Na-bicarbonate, Lonza) which was supplemented with 10% L-cell conditioned medium (source CSF-1) and 10% heat-inactivated Foetal Bovine Serum (FBS, Biochrom, Germany) as growth medium. CSF-1 was required for the differentiation of precursor bone marrow cells to macrophages as well as for the maintenance of the cells. The murine bone marrow derived precursor cells were seeded in six well tissue culture plates (Cellstar, Sigma-Aldrich, USA) and placed in an incubator (37˚C, 5% CO_2) for five days where they could adhere and differentiate into macrophages. The precursor cells were 1 x 10^5 cells per well (2 ml). After five days, undifferentiated cells were washed away, and clean medium was replaced daily until infection was done. After seven days, the macrophages were ready to be infected.

2.3 Infection of Bone Marrow Derived Macrophages

There were three biological replicates, which means that the infection process was done three times with BMDM that came from three different mice for the same time interval. There were two time intervals of

infection, namely a 12h and a 96h post-infection. These timepoints were chosen so that there was an early timepoint of infection (12h) and a late timepoint of infection (96h) involved in the study. The macrophages were infected with two non-pathogenic strains (*M. smegmatis* and *M. bovis* BCG), and a pathogenic strain (M.*tubeculosis* H37Rv). There also was an uninfected control, namely the macrophages that were not infected. *M. smegmatis* was excluded at the 96h time point of Infection.

The Multiplicity of infection or MOI was 2, two bacilli for each macrophage. The syringe settle filtrate (SFF) method was used to break up and remove mycobacterial clumps. After infection the plates were placed in an incubator (37˚C, 5% CO_2). After 4h of infection, the cells were lysed, and the mycobacteria were plated out to observe the percentage uptake and MOI. The MOI was done at 4h because after 4h the maximum uptake of mycobacteria took place. After 4h the six well tissue culture plates were washed three times with phosphate buffered saline (PBS, Lonza, Walkersville, USA) and 0.1% Triton was added to each well. Dilutions of $1 \times 10^{-1} - 1 \times 10^{-4}$ for three technical replicates and of only the unfiltered bacteria were made to validate whether the MOI is 2. The dilutions were plated out on Middlebrook 7H11 agar plates (with 10% OADC enrichment medium and 0.5 % glycerol). The plates were placed in an incubator at 37˚C and the CFUs were counted. *M. smegmatis* was counted after three days while BCG and *M. tuberculosis* was counted after three weeks.

2.4 RNA Extraction

For RNA extraction, the RNeasy Plus Mini kit Cat no. 74134 (Qiagen, Germany) was used. The RNA was extracted from the macrophages after infection of 12h and 96h according to manufacturer's instructions. To remove any DNA from the sample, a gDNA Eliminator column was included in the kit. The RNA samples were send to the Central Analytical Facility, Stellenbosch University. They used an Agilent 2100 Bioanalyzer to assess the quality and quantity of the RNA. Only RNA with an integrity number (RIN) above 9.0 was used for quantitative Polymerase Chain Reaction experiments (qPCR). The RNA extraction was done in three biological replicates for each time interval. The samples were stored at – 80˚C.

2.5 Reverse Transcription

The RNA was converted into cDNA with the QuantiTect Reverse transcription kit Cat no. 05311 (Qiagen, Germany) which included a gDNA wipe out. The volume of RNA that was used to be converted to cDNA was calculated depending on the concentration of each condition and biological replicate. The end concentration of the RNA was 0.5 ng per µl. Optimisation of cDNA calculations for 12h and 96h after infection is included in the appendix. The cDNA was made in the Applied Biosystems 96 well Thermal Cycler. The protocol was followed according to manufacture instructions of the kit. Dilutions were done by adding 80 µl RNase free water and 20 µl of cDNA for each sample. The samples were stored at – 20˚C.

2.6 Quantitative Polymerase Chain Reaction and Analyses

Quantitative Polymerase Chain reaction (qPCR) was done with the mouse gene, *Clec4n.* The qPCR was done in 96 well plates run on a LightCycler 96 (Roche, Germany).

A Fast Start Essential DNA SYBR Green Master mix Cat no. 06924204001 (Roche, Germany) was used for the gene, *Clec4n* with the primer, Clecf10 QuantiTect Primer Assay Cat no. QT00153832 (Qiagen, Netherlands) and Roche H_2O that came with the Green Master Mix was added in volumes according to manufacturer's instructions which resulted in a reaction volume of 20 µl. In the reaction volume 10 µl of the Master mix, 7µl H_2O, 1 µl of the primer and 2 µl of the sample was included for one qPCR reaction.

The reference genes employed were *β-2-Microglobulin* with the primer B2M QuantiTect Primer Assay Cat no. QT 01149547 (Qiagen, Netherlands) and *Ubiquitin C* with the primer UBC QuantiTect Primer Assay Cat no. QT 00245189 (Qiagen, Netherlands). The amplification procedure included 45 cycles for 95 ˚C for 10 s each, followed by 60˚C for 10s and then 72˚C for 10 s.

The experimental groups included *M. smegmatis*, BCG and H37Rv in the 12h timepoint and excluding *M. smegmatis* in the 96h time point with the uninfected as control. There were three biological replicates and two technical replicates for each condition.

The samples of each time point were done on two different 96 well plates and a cDNA Calibrator was included in the plates. To be certain that no DNA contamination was included both for the conversion to cDNA and during qPCR, a non-reverse transcription control as well as a negative control was added respectively. The Roche lightCycler software version 1.1 program was used for qPCR analysis, were relative quantification was calculated, with *B2M* and *UBC* as reference genes, calibrator cDNA as run calibrator, and control (BMDMs with no infection) as study calibrator. Data visualization was normalized. The normalized ratio data was used for Prism. The relative expression which was generated by the software uses the delta delta Ct method. The qPCR was done according to the MIQE guidelines, which are included in the appendix. (Bustin et al., 2009).

The qPCR results were analysed in GraphPad Prism software, where statistical analyses were applied. A one-way ANOVA (and Nonparametric) test was done, with a Bonferroni (compare all pairs of columns) post-test. The significance level was Alpha = 0.05 (95% confidence intervals).

2.7 Protein Extraction

After infection at 12h and 96h, protein was isolated. The wells were washed three times with Phosphate buffered saline (PBS) and then treated with RIPA buffer (Radioimmunoprecipitation assay buffer) which included 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 50 mM Tris (pH 8). The proteins were syringed and filtered (Acrodisc LC 13 mm Syringe Filter with 0.2 µm PVDF Membrane, Sigma Aldrich, USA). The protein samples were stored at -80˚C.

2.8 Protein Quantification

The protein quantification was done with the Bradford Assay. A standard curve was made by adding 900 µl of the quick start Bradford x 1 dye reagent and then a dilution of 10% Bovine Serum Albumin (BSA) cat no: HD14-4 (Qiagen, USA) for 0,10,20,40,60,80 and 100 µl and each dilution is filled up with dH_2O to become

1ml. The 0 volume of BSA was used as a blank. The cuvettes were mixed by hand. The OD_{595} was taken off each dilution with a MRC spectro UV-16 spectrophotometer.

The quick start Bradford x1 dye reagent cat no: 500-0205 (Bio-Rad, USA) of 900 µl was added in a cuvette with 95 µl of dH_2O and 5 µl protein sample. The cuvettes were mixed by hand and the OD_{595} is taken of each sample with MRC spectro UV-16 spectrophotometer.

The calculations were done on Excel to create a standard curve. According to the standard curve protein concentration, the volume for each sample and XT sample buffer, 4X, Bio-Rad were calculated from the standard curve and then added together.

2.9 SDS-PAGE and Western Blots

Samples were prepared by adding the ratio of sample buffer to protein according to the calculated ratio of the standard curve, into 1.5 ml tubes. Before proteins were added, it thawed slowly while kept on ice and were vortexed. After proteins were added, tubes were placed in a heating block at 95˚C for 5 min. Tubes were centrifuged for 1000 rpm for 2 min.

Precast Mini-Protean TCX Gels (Bio-Rad, USA) (10 wells) was loaded with the Precision Plus Protein™ Dual Xtra Prestained Protein ladder cat no 1610377 (Bio rad, USA). In each well 20 µg of sample was loaded. The running Buffer was 10x Tris-Glycine-SDS Buffer (Bio-Rad, USA). The SDS-PAGE was run on 200 Volt. The process was duplicated for two different antibodies. The gels were put on a Trans-Blot Turbo, Mini Format, 0.2 µm PVDF membrane, Bio-Rad into a Trans Blot Turbo (Bio-Rad, USA) for 7 min.

The membranes were washed in x10 TBS (with 1% Tween 80) for 5 min on a LABsmart shaker. It was then washed in 5% Milk powder (Diluted in TBS-Tween 80) for 1 hour on a LABsmart shaker. The membranes were placed in a 50 ml tube with the primary antibody, anti-mDectin-2α, Affinity Purified Goat IgG, cat no AF1525 (R&D Systems, Minneapolis, USA) and reference primary antibody, GAPDH, mouse monoclonal IgG, Anti-

Goat (Santa Cruz Biotechnology, Dallas, USA), with the ratio 1:1000 of Antibody to TBS-Tween 80 and was left overnight on a roller at 4˚C.

The next day the membranes were washed 3 times in TBS-Tween 80 buffer. The membranes were added in a 50 ml tube with the secondary antibody, Donkey anti-Goat IgG-HP (Santa Cruz Biotechnology, Dallas, USA), with 1 µl of antibody in 5 ml of TBS-Tween 80.

Chemiluminescence substrate (250 µl Clarity Max Western ECL Substrate enhancer solution (Bio-Rad, USA) and 250 µl Clarity Max Western ECL Substrate peroxide solution (Bio-Rad, USA)) was added on the section where the band was and after 10 seconds the band was visualised on the Gel-doc with Lab Image software, Bio-Rad.

2.10 Estimating number of cells

A test was done with THP1 Human macrophage cell line (ATCC TIB- 202) to conclude the number of cells added into each well of a 96 well plate (Cellstar, Sigma-Aldrich, USA) that will deliver an 80-90% confluency. THP1 cells were cultured in RPMI- 1640 (containing L-glutamine and Na-bicarbonate, Lonza) with FBS (Biochom, Germany) as growth medium and PMA (Phorbol 12 myristate 13-acetate 1µl per 1 ml of medium).

In a paper by Robinson, M.J et al, the blocking of Dectin-2 receptor with anti Dectin-2 was done on BMDC (Bone marrow dendritic cells). In the study, 1 x 10^5 BMDC was cultured overnight in a 96 well plate. (Robinson et al., 2009).

The calculation of this study was based on a concentration of 5 x 10^5 per 1 ml (for 1 x 10^5 cells in 96 well plate with which is about 200 µl a well). Different number of cells were experimented with to establish what would be the ideal number of cells to start with in order to deliver the best confluency.

Table 2.1 Different THP1 cell number to observe the best confluency in a 96 well plate. PMA (Phorbol 12 myristate 13-acetate) RPMI (Roswell Park Memorial Institute).

Test	Cell Number	Volume of cells	Volume of PMA and RPMI medium	Total volume per well
1	20 000	40 µl	260 µl	300 µl
2	40000	80 µl	220 µl	300 µl
3	50 000	100 µl	200 µl	300 µl
4	75 000	150 µl	150 µl	300 µl
5	100000	200 µl	100 µl	300 µl

After a week the confluency was observed under a microscope and it was found that beginning with 50 000 cells had the best confluency. After a week, the cells increased 10-fold, generating 5 x 10^5 cells.

2.11 Blocking the Dectin-2 Receptor

Six conditions were included in this experiment: 1) Uninfected BMDMs with anti-Dectin-2, 2) Uninfected BMDMs with an Isotype control, 3) Uninfected BMDMs with no antibody, 4) H37Rv infected BMDMs with anti-Dectin-2, 5) H37Rv infected BMDMs with an Isotype control and 6) H37Rv infected BMDMs with no antibody.

In the paper by Robinson, M.J et al, a concentration of 10 µg per ml was used for anti-Dectin-2 and the isotype control when it was added to the BMDCs. The anti-Dectin-2 and isotype control was given 1-3 hours to bind to the receptors on the cell and kept on ice (4˚C). An Isotype control was included to insure that no non-specific binding occurred with the constant region of the IgG antibody.

BMDMs were extracted and seeded as discussed earlier in 2.2. A concentration of 5 x 10^5 cells per ml was plated out in 96 well plates and incubated at 37˚C, 5% CO_2. After a week the cells where treated according to the six different conditions.

	1	2	3	4	5	6	7	8
A	UA	UA	UA		HA	HA	HA	
B								
C	UI	UI	UI		HI	HI	HI	
D								
E	UN	UN	UN		HN	HN	HN	
F								

Figure 2.1 96 well plate layout of the six conditions 1) UA (Uninfected anti-Dectin 2, 2) UI (Uninfected Isotype control, 3) UN (Uninfected No antibody, 4) HA (H37Rv anti-Dectin, 5) HI (H37Rv Isotype), 6) HN (H37Rv No antibody).

BMDMs were treated with a concentration of 10 µg per ml, along with RPMI-1640, supplemented with FBS and CSF. The plates were then left on ice for 2 hours.

The plates were washed x 3 with RPMI-1640 medium. Three of the conditions BMDMs were infected with H37Rv in the same way as discussed in 2.3. Infection of BMDMs were only done with H37Rv because of the limited amount of the anti-Dectin-2 antibody. After 12h and 96h of infection, the supernatant was collected and syringed with 1ml syringe and filtered (Acrodisc LC 13 mm Syringe Filter with 0.2 µm PVDF Membrane, Life Sciences). The supernatant samples were stored at -80˚C . The experiment was done for three biological replicates and it also included three technical replicates for each experiment.

2.11.1 Colony Forming Units

After 12 and 96 hours respectively, the BMDMs infected with H37Rv, were washed three times with RPMI-1640. To lyse the cells, 100 µl of 0.1 % Triton was added to each well that were infected with H37Rv. Serial dilutions were made ($1 x10^{-1} – 1 x 10^{-4}$) with 7H9 medium and plated out on Middelbrook 7H11 medium supplemented with 10 % OADC. After 3 weeks CFU (Colony Forming Units) were counted and compared with the three different conditions: 1) BMDMs infected with H37Rv with Anti-Dectin-2, 2)BMDMs infected with H37Rv with Isotype control, 3)BMDMs infected with H37Rv with no antibody. The experiment was done for three biological replicates and it also included three technical replicates for each experiment. The CFUs at 12h and 96h were presented in a histogram graph with a ratio error bar, where a one-way ANOVA (and Nonparametric) test was done, with a Bonferroni (compare all pairs of columns) post-test. The significance level was Alpha = 0.05 (95% confidence intervals).

2.11.2 ELISA of TNFα and IL-10

Enzyme-linked immunosorbent assay (ELISA) was performed for two cytokines that included TNF-α and IL-10. ELISA was done with the Mouse TNF-α ELISA MAX™ Dulux Set (Cat no 430904, Biolegend, USA), and Mouse IL-10 ELISA MAX™ Duluxe Set (Cat no 431414, Biolegend, USA), according to manufacturer's instructions. The supernatant was collected from the macrophages after infection of 12h and 96h according to manufacturer's instructions. The experiment was done for three biological replicates for each experiment and 2 technical duplicates. The ELISA plates were read on a Versa Max microplate reader at a wavelength of 450 nm and 750 nm.

ELISA results were analysed in Excel and Graphpad Prism. The 570nm absorbance values were subtracted from the 450nm absorbance values before the blank absorbance values were subtracted from the standards and the sample values to get the true OD values. In Prism a standard curve was drawn with a nonlinear regression curve fit. The unknowns were interpolated from the standard curve (second order polynomial). From there the sample concentrations (pg per ml) were presented in a histogram graph with a ratio error

bar, where a one-way ANOVA (and Nonparametric) test was done, with a Bonferroni (compare all pairs of columns) post-test. The significance level was Alpha = 0.05 (95% confidence intervals).

CHAPTER 3: Results

3.1 Gene Expression

3.1.1 The MOI (Multipicity of Infection)

After 4h of infection for three biological replicates, the BMDMs were washed with RPMI. Triton (0.1%) was added to lyse the BMDMs and release the mycobacteria that were taken up. CFU counts were done to calculate the MOI for infection. The MOI was expected to stay consistent for the experiment to be reproducible. The MOI was calculated by the number of BMDMs (2x 10^6) divided by the number of mycobacteria that were taken up. The mean of the MOIs in all the conditions and biological replicates was determined to be about 1.446 and the SEM 0.0833.

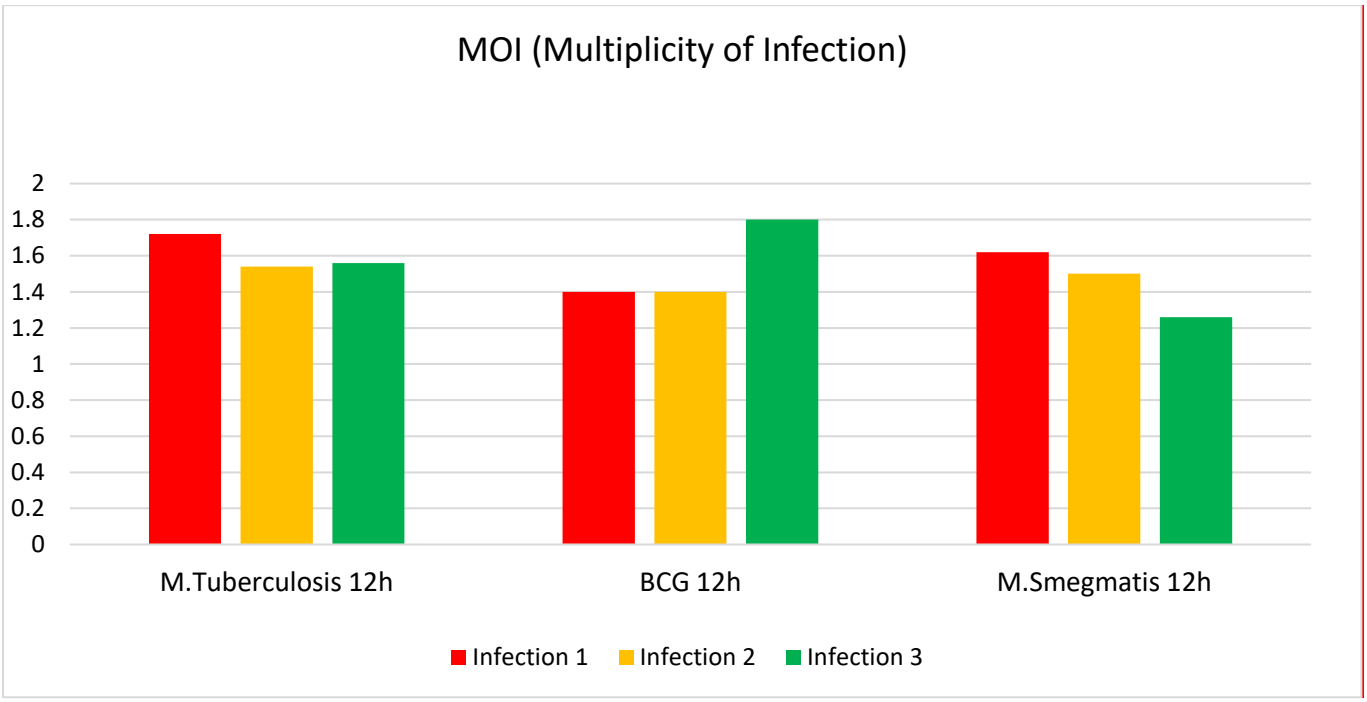

Figure 3.1 The MOI was taken at 4h for all infections. The strains and different biological replicates had an MOI close to 2 and MOI was fairly consistent in the different strains and biological replicates.

3.1.2 RNA Quality and Quantity

The RNA samples were sent to the Central Analytical Facility (CAF) of Stellenbosch University to assess the quality and quantity of it. This is a summary of the results that were generated by the Agilent 2100 Bioanalyzer. All qPCRs were done based on these values. It included the RNA concentration and RIN (RNA integrity number) values. All RNAs extracted at 12h exhibited an RIN value of 10, indicating the highest quality RNA possible. For the 96h timepoint, only 1 RNA sample had a lower RIN value of 7.2. The concentration of all RNA samples ranged from about 300 to 700 µg per µl for the 12h RNA samples. For the 96h samples the RNA concentration ranged from about 100-300 µg per µl. Based on these results, I continued with cDNA synthesis, followed up with qPCR.

Table 3.1 The RIN of RNA Extracted at 12h

12 Hours RNA (ng per µl)						
Strain	Infection 1		Infection 2		Infection 3	
	Concentration	RIN	Concentration	RIN	Concentration	RIN
H37Rv	303	10	273	10	547	10
M. bovis BCG	333	10	434	10	648	10
M. smegmatis	298	10	393	10	543	10
Uninfected	354	10	263	10	692	10

Table 3.2 The RIN of RNA extracted at 96h

96 Hours RNA (µg per ml)						
Strain	Infection 1		Infection 2		Infection 3	
	Concentration	RIN	Concentration	RIN	Concentration	RIN
H37Rv	276	10	223	10	259	7.2
M. bovis BCG	267	10	233	10	211	10
Uninfected	090	10	111	10	287	10

3.1.3 Gene Expression

The qPCR results were analysed in GraphPad Prism software where statistical analysis was applied. A One-way ANOVA (and Nonparametric) test was done, with a Bonferroni (compare all pairs of columns) post-test. The significance level was Alpha = 0.05 (95% confidence intervals).

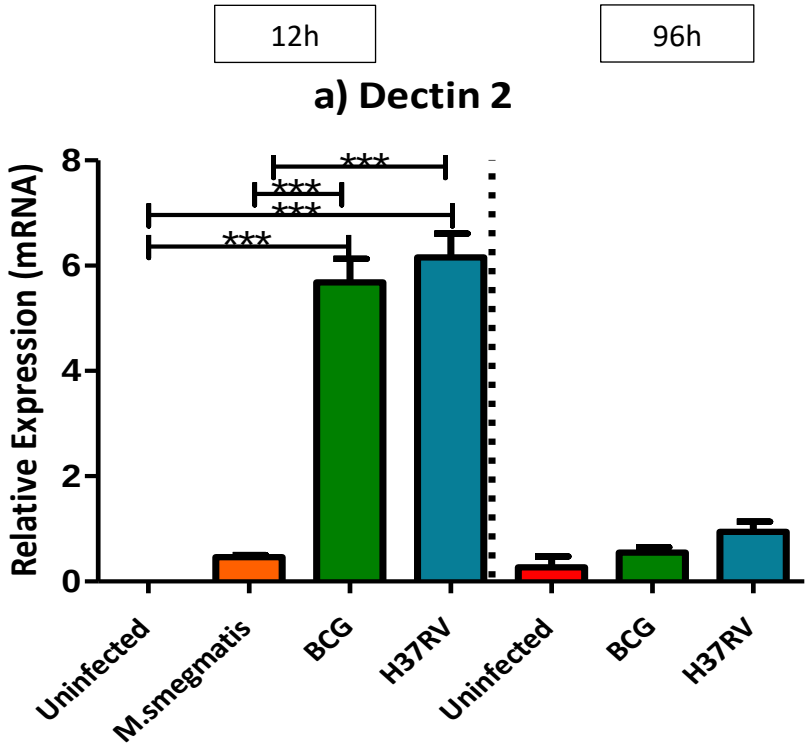

Figure 3.2 Dectin-2 gene expression represented in a histogram after 12h and 96h of infection. At 12h after infection a significant upregulation has occurred in BMDMs infected with surviving mycobacteria compared to BMDMs infected with non-surviving mycobacteria. At 96h after infection Dectin-2 expression decreased in all strains and no significant difference occurred between the different strains.

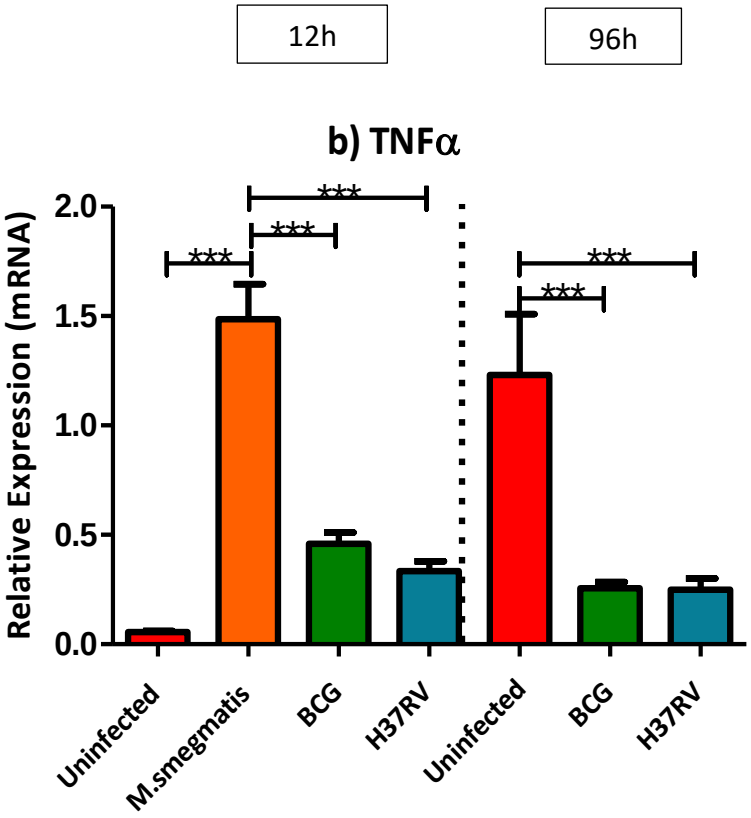

Figure 3.3 TNF α gene expression represented in a histogram after 12h and 96h of infection. At 12h after infection with *M.smegmatis* a significantly upregulation occurred compared to infection with the other strains and uninfected. At 96h after infection the uninfected BMDMs had a significant upregulation compared to the infected BMDMs.

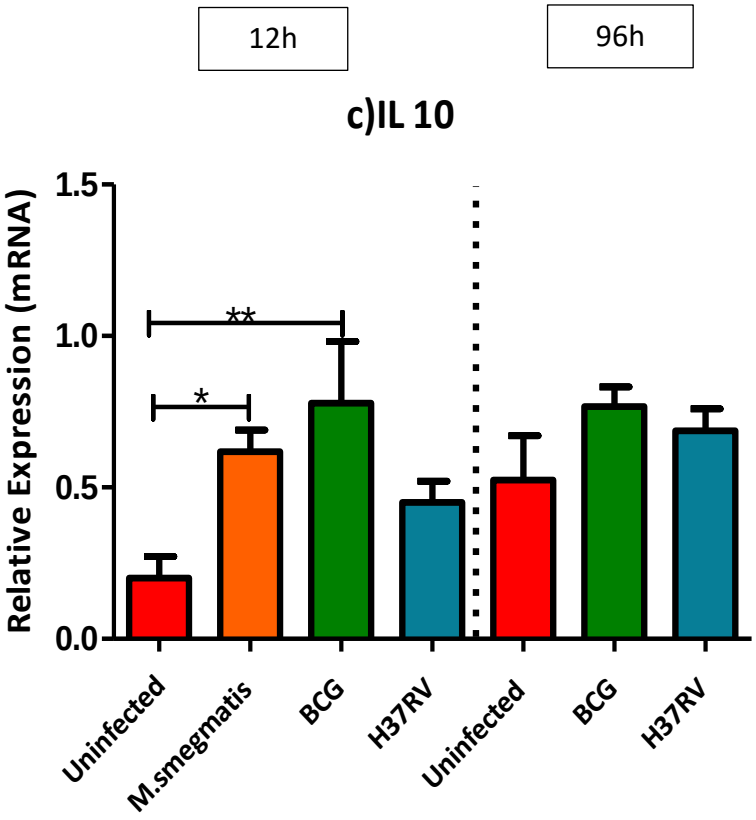

Figure 3.4 IL 10 gene expression represented in a histogram after 12h and 96h of infection. At 12h after infection with BCG and *M.smegmatis* a significant upregulation occurred compared to infection with the uninfected BMDMs. At 96h upregulation occurred in all BMDMs including infected and uninfected.

For the 12h time point the four conditions included: BMDMs that were uninfected, and infected with respectively *M. smegmatis*, *M. bovis* BCG and H37Rv. At the 96h time point, BMDMs infected with *M. smegmatis* was excluded since *M. smegmatis* is killed inside the host macrophages after 24h and a problem with extracellular growth in RPMI-1640 medium was encountered.

In figure 3.2, after 12h after infection upregulation occurred in BMDMs that were infected and uninfected. A significant upregulation did occur in BMDMs infected with surviving mycobacteria compared to BMDMs

infected with non-surviving mycobacteria. The three *** indicate a significant difference with P value ($p \leq 0.0010$), with a 0.1 % that this difference occurred due to chance. At 96h fold change decreased with BMDM infection except for the uninfected that increased, but no significant difference occurred.

In figure 3.3 and 3.4, gene expression was done on cytokines that are known to be induced when the Dectin-2 receptor recognize *M.tuberculosis*, which were the cytokines TNF α and IL 10 to obtain an overview of the cytokine release after 12h and 96h infection with different mycobacteria strains in BMDMs.

In figure 3.3, expression of TNF α occurred in all BMDM conditions. After 12h of infection, BMDMs infected with *M.smegmatis* significantly upregulated TNF α compared with uninfected BMDMs and BMDMs that were infected with the other mycobacterium strains. After 96h of infection the uninfected BMDMs significantly upregulated TNF α compared to the infected BMDMs.

In figure 3.4, expression of IL 10 occurred in all BMDM conditions. After 12h of infection there was a significant upregulation in BMDMs infected with M.smegmatis and BCG when compared to the uninfected BMDMs. The two ** indicate a significant difference with p value ($p \leq 0.01$), with a 1 % that this difference occurred due to chance. One * indicate a significant difference with p value ($p \leq 0.05$), with a 5 % that this difference occurred due to chance. After 96h of infection no significant difference occurred between the BMDM conditions.

3.2 Protein Expression

To assess whether the qPCR data on *Clec4n* correlates with an increase in the protein levels of Dectin-2, protein was extracted from BMDMs that have been infected with *M. smegmatis*, *M. bovis* BCG and H37Rv as well as uninfected BMDMs after 12h and 96h respectively, (excluding *M. smegmatis* for 96h) for western blot. The reference antibody, GAPDH was used (*gapdh* is a housekeeping gene) to determine whether the

protein extraction, and western blots have been done correctly to reliably quantify the level of Dectin-2. Unfortunately the inconsistency of the GAPDH protein expression between the different conditions and biological replicates occurred therefor no conclusions could be drawn from this data. The Full western blot is included in the appendix.

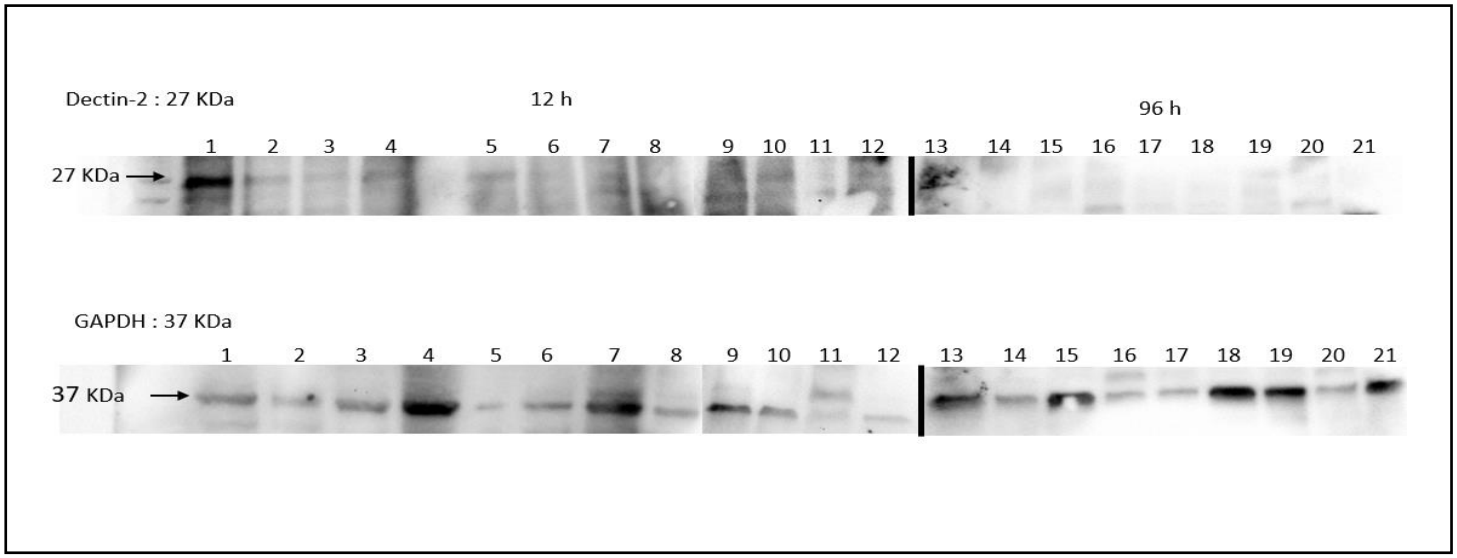

Figure 3.5 Western blot for Dectin-2 protein and GAPDH protein. The numbers were labelled accordingly, 1) 12 hours experiment 1 uninfected, 2) *M. smegmatis* , 3) BCG, 4) H37Rv, 5) experiment 2 uninfected, 6) *M. smegmatis*, 7) BCG, 8) H37Rv, 9) experiment 3 uninfected, 10) *M. smegmatis*, 11) BCG, 12) H37Rv, 13) 96 hours experiment 1 uninfected, 14) BCG , 15) H37Rv, 16) experiment 2 uninfected, 17) BCG , 18) H37Rv, 19) experiment 3 uninfected, 20) BCG, 21) H37Rv.

3.3 Blocking the Dectin-2 receptor

3.3.1 Survival of pathogenic mycobacteria

Six conditions were included in this experiment: 1) Uninfected BMDMs with anti-Dectin-2, 2) Uninfected BMDMs with an Isotype control, 3) Uninfected BMDMs with no antibody, 4) H37Rv infected BMDMs with

anti-Dectin-2, 5) H37Rv infected BMDMs with an Isotype control and 6) H37Rv infected BMDMs with no antibody.

The conditions that were infected with H37Rv, were plated out on 7H11 Middlebrook medium with OADC enrichment and incubated at 37˚C, after a 12h and 96h incubation. After 3-4 weeks incubation, the plates were counted for CFUs. As can be observed from Table 3.4 the treatment of the cells with Dectin-2 antibody decreased the amount of H37Rv at 12h post infection compared to the isotype antibody and no antibody controls. However, no differences in growth are observed in CFUs from 12h to 96h post infection.

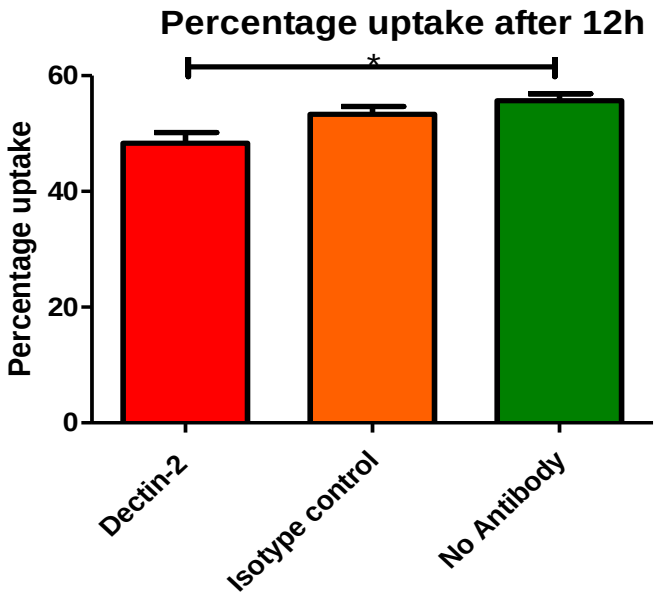

Figure 3.6 represents the percentage uptake after 12h of Infection in BMDMs infected with H37Rv and treated with either the anti-Dectin-2 antibody, the isotype control or have no antibody treatment. There was found to be a significant decrease in the BMDMs treated with the anti-Dectin-2 antibody compared to BMDMs and no antibody treatment.

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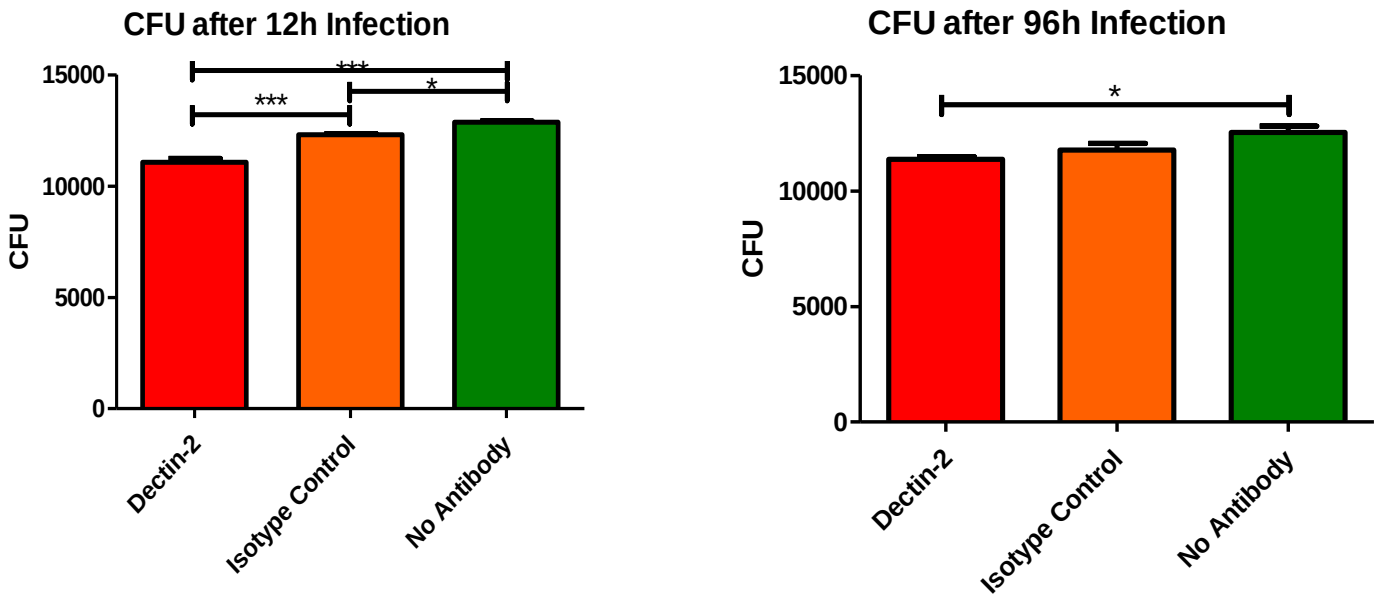

Figure 3.7 CFU count and percentage uptake of pathogenic mycobacteria (H37Rv) was compared in BMDMs that were treated with a Dentin-2 antibody, Isotype control and no antibody after 12h and 96h infection to determine if there is any change in CFUs between the conditions.

3.3.2 Cytokine release of TNF α and IL-10

Supernatant was collected in all conditions after 12h and 96h infection. ELISAs were done to estimate the concentration (pg per ml) of TNF-α and IL-10.

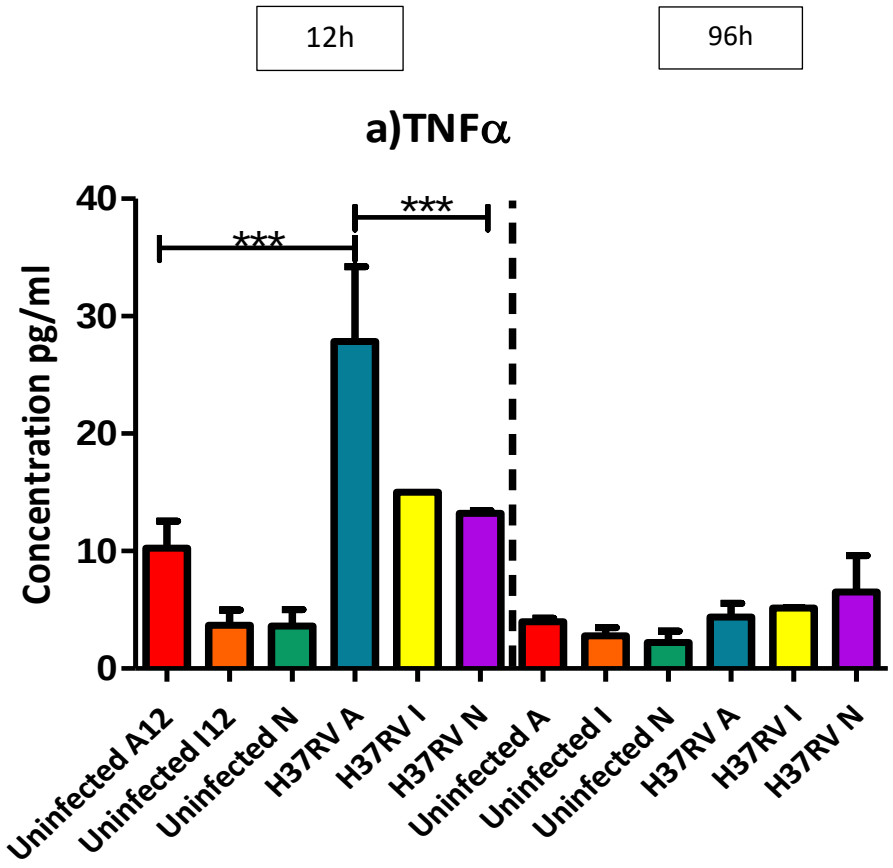

Figure 3.8 TNF α production represented in a histogram after 12h and 96h infection with H37Rv in BMDMs. After 12h of infection, BMDMs infected with H37Rv and treated with anti-Dectin-2 antibody significantly upregulated TNF α compared with BMDMs infected with H37Rv and no antibody treatment as well as uninfected BMDMs with anti-Dectin-2 antibody treatment. After 96h of infection, production of TNF α decreased in all conditions and no significant difference occurred. (A = Anti-Dectin-2 antibody, I = Isotype control, N= No antibody treatment)

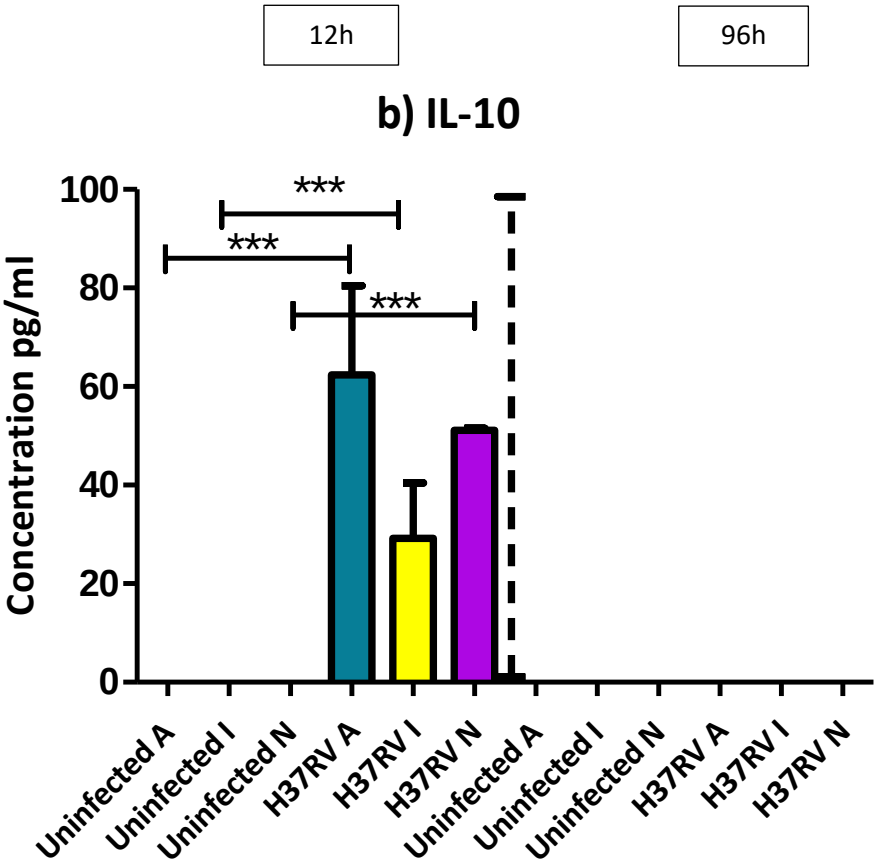

Figure 3.9 IL 10 production represented in a histogram after 12h and 96h of infection with H37Rv in BMDMs. After 12h of infection IL 10 production occurred in the BMDMs that were infected, but Il 10 production in the uninfected BMDMs were lower than the limit of detection. No significant difference occurred between the infected BMDMs. After 96h of infection, IL-10 production was lower than the limit of detection. (A = Anti-Dectin-2 antibody, I = Isotype control, N= No antibody treatment)

In Figure 3.7, after 12h and 96h of Infection all BMDM conditions had TNF α production. At 12h after infection, BMDMs infected with H37Rv and treated with the anti-Dectin-2 antibody had a significant higher production of TNF α compared to BMDMs infected with H37Rv and no antibody treatment as well as compared to uninfected BMDMs treated with the anti-Dectin-2 antibody. The uninfected BMDMs that were treated with the anti-Dectin-2 antibody had a higher production of TNF α than the other uninfected conditions. After 96h of infection a decrease was observed in all conditions and no significant difference occurred between these conditions.

In figure 3.8, after 12h of infection, only the infected BMDMs had a IL 10 release. After 96h of infection the IL 10 release was lower than the limit of detection.

CHAPTER 4: Discussion

Although TB Drugs that are now found on the market have been able to cure many TB patients, multi-drug resistant and extremely drug resistant mycobacteria have become a threat for TB patients worldwide (WHO, 2017). An alternative way of combatting TB would be to increase the host immune response by host directed therapeutics rather than focussing only on developing drugs targeting the mycobacteria. As discussed previously pathogenic mycobacteria are able to avoid bactericidal host defences and have developed ways to prevent being eliminated by an active immune response (Eht and Schnappinger, 2009).

In a high throughput RNA sequencing study, several genes of the host cells (BMDMs) were compared in expression and significance between mycobacteria (H37Rv and *M. bovis BCG*) that are able to survive in the macrophage and mycobacteria (*M. smegmatis*) that are not. It was found that the expression of one of the genes, *Clec4n* was significantly higher in the BMDMs that were infected with the H37Rv and *M. bovis* BCG when compared to *M. smegmatis* and the uninfected macrophages. It is possible that *Clec4n* (Dectin-2) plays a role in the survival of pathogenic mycobacteria.

4.1 Gene Expression

4.1.1 The MOI (Multiplicity of Infection)

The aim was to achieve a MOI of 2. The MOI was expected to stay consistent for the experiments to be reproducible. MOI was calculated by the number of BMDMs (2×10^6) infected, divided by the number of mycobacteria that were taken up by the cells.

As mentioned in the methods, *M. smegmatis* was excluded at 96h time point of Infection. *M. smegmatis* was killed after 24h of infection in the macrophages (Kuehnel et al., 2001). *M.smegmatis* is a fast-growing mycobacteria that starts to grow extracellularly in the RPMI-medium and acts as contamination to the

BMDMs. Infection with *M. smegmatis* was excluded for qPCR and western blot experiments at a 96 h timepoint in this study.

In figure 3.1, with calculations to obtain a MOI of 2, the MOIs did not reach 2, but were close to 2 and fairly consistent between different conditions and biological replicates. Possible reasons for that fact that the observed MOI that was not equal to 2 could be because the mycobacteria was not grown in Tween-80. Tween-80 prevents clumping of mycobacteria (Sani et al., 2010), which means without Tween-80, even with the SSF, this could possibly still be a factor that influences the uptake of mycobacteria by BMDMs. It was also found that even after counting the cells with a haemocytometer, calculating the volume that needed to be added to each well and mixing before adding the volume, still resulted in a difference in the confluency between wells when observed under the microscope.

4.1.2 RNA Quality and Quantity

The RNA concentration (µg per ml) at 12h in table 3.1 and 96h in table 3.2 was high enough to convert to cDNA and use for qPCR. The RIN (RNA Integrity Number) was calculated by the ratio area under 18S and 28S RNA peaks to the total area under the graph of an electropherogram. The RIN value is an indication of the amount of RNA that has been degraded (Schoeder et al., 2006). When the RIN value is 10, all the RNA is still intact. It was found that the RNA after 12h infection (table 3.2) and 96h infection (table 3.3), all had RIN values of ten, expect for the BMDMs that were infected with H37Rv after 96h of infection which had a RIN value of 7.2. This can be due to the conditions of the cells, growth of infection may have caused stress of the cells, the way samples were handled and sample measurement. The high difference in RNA concentration between the 12h and 96h time point could maybe be due to the BMDMs that were washed more times at 96h, which could lead to some of the BMDMs getting lost during washing.

4.1.3 Gene Expression

4.1.3.1 *Clec4n* gene expression

Gene expression of Dectin-2 at a 12h and 96h timepoint of infection with *M.smegmatis*, BCG and H37Rv in BMDMs is presented in figure 3.2. After 12h of infection, BMDMs that were infected with BCG and H37Rv significantly upregulated Dectin-2, compared to BMDMs infected with *M.smegmatis* as well as uninfected BMDMs. These results validate the results that were found in the RNA sequencing data. Because H37Rv and BCG is able to survive in the macrophage (Meena and Rajni, 2010) and *M.smegmatis* dies after 24h (Kuehnel et al., 2001), these results can suggest that the Dectin-2 receptor may influence the survival of H37Rv and BCG. It is still unknown in the literature whether Dectin-2 has an influence on the survival of mycobacteria, however it was found in Dectin-1 deficient mice that there was a reduction in pulmonary bacilli loads but no significant changes in pulmonary pathology, cytokine levels or the ability of mice to survive infection with *M.tuberculosis* (Marakalala et al., 2011). After 96h a decrease occurred in the infected BMDMs. This can indicate that Dectin-2 plays a more important role at the early stages of infection. It is possible that after 96h of infection, other receptors that *M.tuberculosis* will bind to in a higher abundancy were higher expressed and that less stimulation through *M.tuberculosis* to the Dectin-2 receptor led to a decrease in Dectin-2 expression. At 96h the expression of Dectin-2 in BMDMs with non-surviving mycobacteria could not be determined since *M.smegmatis* was killed after 24h of infection in the macrophages. This made it difficult to compare non-surviving mycobacteria to surviving mycobacteria and determine the influence of Dectin-2 at 96h after infection. Eliminating the *Clec4n* gene or the Dectin-2 receptor will give more insight in whether this gene plays a role in the survival of these strains.

4.1.3.2 Cytokine Gene Expression of *TNF* α

After binding of ManLaM to the Dectin-2 (*Clec4n*) receptor on DCs or Macrophages, Dectin-2 induces the production of pro- and anti-inflammatory cytokines, that include TNF α and IL-10 (Marakalala and Ndlovu, 2017). The gene expression of *TNFα* and *IL-10* was observed to obtain an overview of the cytokines TNF α and IL 10 expression in BMDMs after 12h and 96h infection with different mycobacteria strains in BMDMs.

In figure 3.3, the *TNF α* gene was found to be upregulated in each of the conditions, however after 12h of infection, *TNF α* expression was significantly upregulated in BMDMs infected with *M.smegmatis* compared to uninfected BMDMs and BCG infected BMDMs. This high expression in BMDMs infected with *M.smegmatis* is to be expected since *M.smegmatis* is not able to survive in the macrophage. A high TNF α likely contributes to the killing of *M.smegmatis*.

After 96h of infection, uninfected BMDMs had a high increase of *TNF α* compared to the 12h infection and a significant higher expression of *TNF α* compared to both the infected BMDMs. This result was not expected, in the ELISA assays the uninfected with no antibody treatment had a higher cytokine response than the infected BMDMs with no antibody treatment. The observation of the cytokine release is a better representation of the cells immune response in comparison with gene expression. Gene expression does not always correlate with protein expression. Post translational modification may lead to a different protein expression as would be expected when compared to the gene expression.

4.1.3.3 Cytokine Gene Expression of *IL 10*

In figure 3.4, the *IL 10* was found to be upregulated in all the BMDM conditions after 12h and 96h of infection. After 12h of infection, BMDMs infected with BCG and *M.smegmatis* were significantly upregulated compared to the uninfected BMDMs. After 96h of infection no significant difference occurred.

Based on the results, the *TNF-α* and *IL-10* gene was not relatively expressed in the same way as the *Clec4n* gene between the different conditions. This means that *Clec4n* expression does not have a direct effect on the expression of TNFα and IL-10.

4.2 Protein Expression

In figure 3.5, the GAPDH gene (which is a housekeeping gene) was found to be present on the blot in each of the samples but the concentrations were not consistent. The optimising of western blots included: different transmembrane blotting time, different blocking time, different concentrations of primary and

secondary antibodies, and repeating protein extraction. Low concentrations of protein could have led to inconsistency.

The Dectin-2 protein were found to be expressed in some of the samples, such as the uninfected BMDMs, but some samples did not show any bands. Anti-mDectin-2α, Affinity Purified Goat IgG, is a polyclonal antibody and it could be possible that more non-specific binding took place that led to faint or no bands. No inferences or conclusions can be drawn from this data.

4.3 Blocking the Dectin-2 receptor

To achieve a better understanding in the role that Dectin-2 plays during infection of pathogenic mycobacteria in mouse macrophages, the Dectin-2 receptor was blocked with an anti-Dectin-2 Antibody, to observe the effects on mycobacterial survival and percentage uptake.

4.3.1 Difference in survival of pathogenic mycobacteria

After 12h of infection the percentage uptake was observed. In figure 3.6, there was a significant decrease in the uptake of H37Rv in BMDMs treated with the anti-Dectin-2 antibody compared to the BMDMs with no antibody treatment. A change in percentage uptake also occurred between BMDMs with anti-Dectin-2 antibody treatment and BMDMs with the isotype treatment.

In figure 3.7, after 12h of infection there was a significant decrease in the CFU in the BMDMs treated with anti-Dectin-2 antibody compared to CFUs in BMDMs with isotype control treatment and no antibody treatment. After 96h the significant decrease of CFUs in BMDMs treated with the anti-Dectin-2 antibody compared to the CFUs in BMDMs with no antibody treatment was maintained. When the CFUs of 12h of infection was compared to the CFUs of 96h there was no change in the survival or growth of the

mycobacteria. The results can indicate that Dectin-2 may influence the percentage uptake of H37Rv but not the survival.

4.3.2 Cytokine release of TNFα and IL-10

ELISA was performed on supernatants collected after a 12h and 96h post-infection, with the following six conditions: 1) Uninfected BMDMs with anti-Dectin 2, 2) Uninfected BMDMs with an Isotype control, 3) Uninfected BMDMs with no antibody, 4) H37Rv infected BMDMs with anti-Dectin 2, 5) H37Rv infected BMDMs with an Isotype control and 6) H37Rv infected BMDMs with no antibody. The concentration (pg per ml) of TNFα and IL-10 was calculated with a standard curve. Conditions that only had one variable were compared with each other.

In figure 3.8, in both timepoints ,TNF α release occurred in all the different conditions. The BMDMs that were infected with pathogenic mycobacteria (H37Rv) after 12h time point of infection, all had a higher concentration (pg per ml) compared to the BMDMs that were uninfected, as expected. There was a significant increase of TNF α at the 12h time point of infection between the BMDMs infected with H37Rv and treated with the anti-Dectin-2 antibody, and between the BMDMs that were infected with H37Rv and not treated with the antibody. This increase can be because blocking the Dectin-2 receptor causes the increase of mycobacteria binding to other receptors that also leads to a high TNF α release. A higher release of TNF α can lead to killing pathogenic mycobacteria. Uninfected BMDMs had a higher production of TNF α release than the uninfected BMDMs treated with the isotype control and no antibody treatment. It can be concluded that binding of the antibody stimulated the Dectin-2 receptor, that leads to a higher production of TNF-α.

At the 96h time point after infection, the production of TNF-α had decreased in all the BMDMs conditions. No significant difference in production was found between conditions at this time point.

In figure 3.9, at the 12h time point of infection, only the BMDMs that were infected with mycobacteria (H37Rv) had an increased in the release of the IL-10 but no significant difference occurred between these

conditions. In the uninfected BMDMs at 12h post-infection al IL-10 concentrations were below the limit of detection.

At 96h all of the IL-10 concentrations (pg per ml) were found to be below the limit of detection on the standard curve. It can be concluded that blocking the Dectin-2 receptor does not have a significant effect on the IL-10 release during pathogenic mycobacteria post-infection at 12h or 96h of infection respectively. It can be concluded from the 96h post-infection that IL10 does not play a role at the later (96h) stages of infection.

4.4 Future studies

To improve this study and to make it more reliable, experiments such as immunohistochemistry can be done to show that the anti-Dectin-2 Antibody binds to the surface of the BMDMs and not to mutant BMDMs deficient in Dectin-2. This was a drawback in this study, since it could not be proven that the antibody was bound to the Dectin2 receptor. The only way to have some estimate whether the anti-Dectin-2 antibody did bind to the Dectin-2 receptor would be to observe the difference between the conditions of BMDMs that were treated with anti-Dectin2, Isotype control (non-specific binding) and no treatment.

Gene silencing on the *Clec4n* gene (Dectin2) would be a much more sensitive assay. It would eliminate any Dectin-2 receptor and likely have a more robust estimation of the role of Dectin-2 production and activity. It is also possible that the binding of the Dectin-2 antibody can stimulate the receptor. Silencing would eliminate this unwanted side effect.

To broaden the study, including more strains such as *M. smegmatis* and *M. bovis* BCG could be done. Doing so, could make it possible to observe the effect that Dectin-2 has on non-pathogenic strains. It would be advisable to do ELISAs on more cytokines that Dectin-2 is known to effect during mycobacteria infection.

Further, the effects observed in mouse bone marrow-derived macrophages may differ from what would be observed with human blood monocyte-derived macrophages and the human THP-1 cell line. The study

using the anti-Dectin-2 antibody must be repeated with human macrophages to obtain a better insight based on tuberculosis in humans.

4.5 Conclusion

According to the qPCR results *Clec4n* is significantly upregulated, which indicates a possible role in BMDMs of *M. bovis* BCG and H37Rv compared to uninfected and *M. smegmatis*.

When the CFUs of 12h of infection was compared to the CFUs of 96h there was not a change in the survival or growth of the mycobacteria. Its results can indicate that Dectin-2 may influence the percentage uptake of H37Rv but not the survival.

In the ELISA results it was found that there was a significant increase in the release of the TNF α cytokine, in the infected BMDMs treated with the anti-dectin-2 antibody compared to the infected BMDMs with no antibody treatment from which can be concluded that blocking the Dectin-2 receptor causes the increase of mycobacteria binding to other receptors that also lead to a high TNF α release. A higher release of TNF α can lead to killing pathogenic mycobacteria. There was found to be a higher production in TNF α with BMDMs that were not infected with H37Rv but treated with Dectin-2 antibody in comparison with BMDMs with no infection and Isotype control and no antibody treatment. This is found to be an experimental flaw as it would have been expected to have the same amount of TNF-α production in each of these three variables.

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Appendix

Table 1. MIQE checklist for authors, reviewers, and editors.[a]

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D[d]
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE[b] samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C_q of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and y intercept	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D
Inhibition testing (C_q dilutions, spike, or other)	E	r^2 of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C_q variation at LOD	E
Amount of RNA and reaction volume	E	CIs throughout range	D
Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
Temperature and time	E	Data analysis	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C_qs with and without reverse transcription	D[c]	Method of C_q determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C_q or raw data submission with RDML	D

[a] All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.
[b] FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.
[c] Assessing the absence of DNA with a no–reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no–reverse transcription control is desirable but no longer essential.
[d] Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

CDNA Optimisation 12h 22-01-2018

	RNA Concentration (ng per µl)	
1	Uninfected-12-1	432
2	Uninfected-12-2	305
3	Uninfected-12-3	353
4	M.Smegmatis-12-1	298
5	M.Smegmatis-12-2	393
6	M.Smegmatis-12-3	543
7	BCG-12-1	333
8	BCG-12-2	434
9	BCG-12-3	648
10	H37RV-12-1	303
11	H37RV-12-2	273
12	H37RV-12-3	547
13	R179-12-1	387
14	R179-12-2	299
15	R179-12-3	319

	RNA volume (0.5 ng per µl) (µl)					
	Sample	RNA	GDNA	H_2O	MM	Total
1	Uninfected-12-1	1.2	2	10.8	6	20
2	Uninfected-12-2	1.6	2	10.4	6	20
3	Uninfected-12-3	1.4	2	11.6	6	20
4	M.Smegmatis-12-1	1.7	2	10.3	6	20
5	M.Smegmatis-12-2	1.3	2	10.7	6	20
6	M.Smegmatis-12-3	0.9	2	11.1	6	20
7	BCG-12-1	1.5	2	10.5	6	20
8	BCG-12-2	1.2	2	10.8	6	20
9	BCG-12-3	0.8	2	11.2	6	20
10	H37RV-12-1	1.7	2	10.3	6	20
11	H37RV-12-2	1.8	2	10.2	6	20
12	H37RV-12-3	0.9	2	11.1	6	20
13	R179-12-1	1.3	2	10.7	6	20
14	R179-12-2	2.2	2	09.8	6	20
15	R179-12-3	1.6	2	10.4	6	20
16	NTC	0.0	2	12	6	20

	X1	X 17
RT Enzyme	1 µl	17 µl
RT Buffer	4 µl	68 µl
RT Primer mix	1 µl	17 µl

Dilutions: 80 µl of RNase Free Water and 20 µl of cDNA .Calibrator: 10 µl of each sample

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	RNA Concentration (ng per µl)	
1	BCG-96-1	267
2	BCG-96-2	233
3	BCG-96-3	211
4	H37RV-96-1	276
5	H37RV-96-2	223
6	H37RV-96-3	259
7	R179-96-1	196
8	R179-96-2	182
9	R179-96-3	194
10	Uninfected-96-1	90
11	Uninfected-96-2	111
12	Uninfected-96-3	287

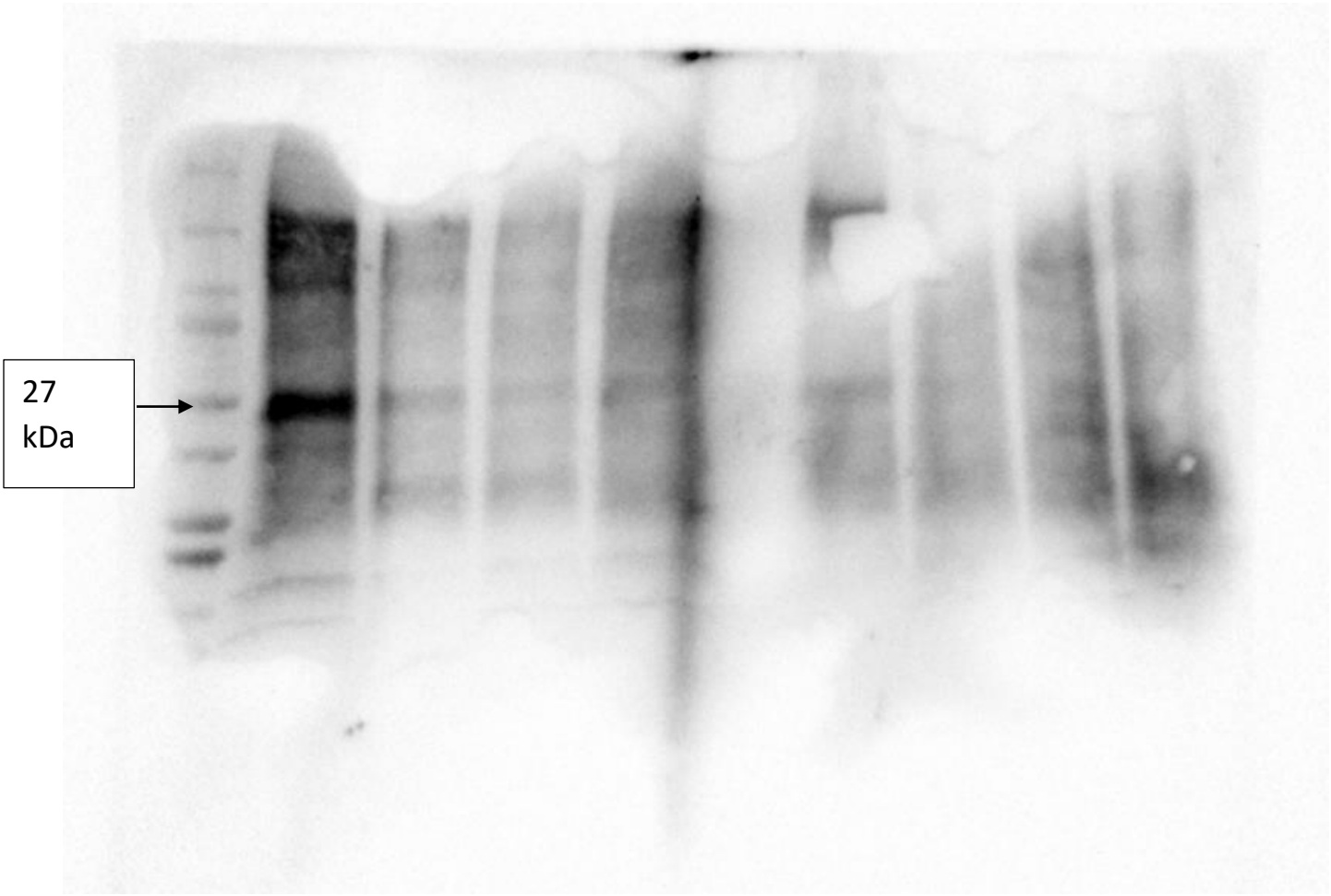
	RNA volume (0.5 ng per µl) (µl)					
	Sample	RNA	GDNA	H_2O	MM	Total
1	BCG-96-1	1.9	2	10.1	6	20
2	BCG-96-2	2.2	2	09.8	6	20
3	BCG-96-3	2.4	2	09.6	6	20
4	H37RV-96-1	1.8	2	10.2	6	20
5	H37RV-96-2	2.2	2	09.8	6	20
6	H37RV-96-3	1.9	2	10.1	6	20
7	R179-96-1	2.6	2	09.4	6	20
8	R179-96-2	2.8	2	09.2	6	20
9	R179-96-3	2.6	2	09.4	6	20
10	Uninfected-96-1	5.6	2	06.4	6	20
11	Uninfected-96-2	4.5	2	06.6	6	20
12	Uninfected-96-3	1.7	2	10.3	6	20
13	Non-Transcription	0.0	2	12.0	6	20
14	Non-Transcription	0.0	2	12.0	6	20

	X1	X 15
RT Enzyme	1 µl	15 µl
RT Buffer	4 µl	60 µl
RT Primer mix	1 µl	15 µl

Dilutions: 80 µl of RNase Free Water and 20 µl of cDNA

Calibrator: 10 µl of each sample

Dectin-2 Western Blot



GAPDH Western Blot